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Development and validation of a high performance liquid chromatography method for the simultaneous determination of aspirin and folic acid from nano-particulate systems

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Abstract

Attention has shifted from the treatment of colorectal cancer (CRC) to chemoprevention using aspirin and folic acid as agents capable of preventing the onset of colon cancer. However, no sensitive analytical method exists to simultaneously quantify the two drugs when released from polymer-based nanoparticles. Thus, a rapid, highly sensitive method of high performance liquid chromatography (HPLC) analysis to simultaneously detect low quantities of aspirin (hydrolyzed to salicylic acid, the active moiety) and folic acid released from biodegradable polylactide-co-glycolide (PLGA) copolymer nanoparticles was developed. Analysis was done on a reversed phase C₁₈ column using a photodiode array (PDA) detector at wavelengths of 233 nm (salicylic acid) and 277 nm (folic acid). The mobile phase consisted of acetonitrile – 0.1% trifluoroacetic acid mixture programmed for a 30 min gradient elution analysis. In the range of 0.1 – 100 µg/mL, the assay showed good linearity for salicylic acid ($R^2 = 0.9996$) and folic acid ($R^2 = 0.9998$). The method demonstrated good reproducibility, intra- and inter-day precision and accuracy (99.67, 100.1%) and low values of detection (0.03, 0.01 µg/mL) and quantitation (0.1 and 0.05 µg/mL) for salicylic acid and folic acid, respectively. The suitability of the method was demonstrated by simultaneously determining salicylic acid and folic acid released from PLGA nanoparticles.

Keywords

aspirin; salicylic acid; folic acid; simultaneous detection; nano-particulate systems; HPLC

Introduction

According to the American Cancer Society, approximately 50,000 people are estimated to die from colorectal cancer (CRC) in 2009, making this disease one of the largest killers in the United States. Current research is shifting the focus of colorectal cancer disease from diagnosis and treatment to chemoprevention (Gill *et al.*, 2005). Chemoprevention aims to prevent the development or recurrence of precancerous lesions and cancers with the use of natural or synthetic agents that reverse, suppress, delay, or prevent carcinogenic progression to invasive disease (Serrano *et al.*, 2004). Preclinical and clinical studies using several well known pharmacologic (e.g., aspirin, sulindac, celecoxib) and non-pharmacologic (e.g., calcium, folic acid, curcumin, vitamins) agents have shown promising results (Viner *et al.*, 2002) in preventing the occurrence of colon cancer. Among these, aspirin and folic acid

have been widely investigated as chemopreventive agents against CRC. Both of these compounds have shown significant reduction in CRC with aspirin exhibiting 40-50% reduction among chronic users of these drugs (Kinzler *et al.*, 1998) whereas folic acid studies demonstrated a 31% reduction in the risk of colorectal adenomas (Giovannucci *et al.*, 1998). Additionally, there is increasing interest in the use of combinations of low doses of chemopreventive agents providing means of obtaining synergistic efficacy and minimized toxicity because promising chemopreventive agents often demonstrate undesirable toxic effects at higher doses otherwise (Reddy *et al.*, 2004).

In order to effectively deliver these combinations of chemopreventive agents at low doses, a nanotechnology-based drug delivery system was developed which was capable of encapsulating aspirin and folic acid within a biodegradable polymer, poly-lactide-(co-glycolide) (PLGA), and targeting directly into the colon for simultaneous controlled release of these agents over time (Kanthamneni *et al.*, 2007). Poly(lactide-co-glycolide) (PLGA) an FDA approved, biodegradable co-polymer, has been widely used as matrix material for the preparation of drug-loaded nanoparticles (Panyam, *et al.*, 2003; Soppimath, *et al.*, 2001). The release of drug from nanoparticles depends on polymer degradation due to hydrolysis, which is governed by the nature of copolymer composition and its molecular weight. PLGA co-polymers are available in different grades (85:15, 75:25, 65:35, 50:50) based on the ratio of lactic acid to glycolic acid present within them, respectively. PLGA 50:50 co-polymer is known to hydrolyze at a faster rate than those containing a higher proportion of polylactic acid such as PLGA 75:25 thus releasing the encapsulated drug at a faster rate than other PLGA co-polymers (Hariharan, *et al.*, 2006). As the amount of lactic acid increases in the co-polymer, drug release slows down due to the increasing relative insolubility of lactic acid in water. Based on these different compositions and molecular weights, delivery systems can be designed to release drug for varying periods of time. In vivo, these polymers undergo hydrolysis and turn into water and carbon dioxide through the citric acid cycle (Jain, 2000).

Nano-particulate drug delivery systems offer several advantages such as controlled and sustained release of drugs, ability to cross the mucosal barriers, decreased intestinal, renal and hepatic clearances, increased apparent half-lives of drugs due to encapsulation and slow release from polymers, enhanced intracellular uptake thereby allowing drug release in different cellular compartments, increased stability and solubility of drugs as well as reduce drug resistance in some human carcinomas (Orive *et al.*, 2005). Additionally, nanoparticles have a large surface area which is indicative of a high interactive potential with biological surfaces (Krishnaiah 2003). Such multi-particulate systems also tend to be more uniformly dispersed in the GI tract and ensure uniform absorption (Rodrigues *et al.*, 1998). Thus, it is apparent that these systems are well-suited for cancer chemoprevention research and studies in this area could be of great potential and practical value.

The quantification of the aspirin and folic acid released from the PLGA nanoparticles is important for the assessment of the chemopreventive effects of these agents against colon cancer. Several studies exist in literature that have developed HPLC assays to detect a host of chemical compounds including aspirin and folic acid. These studies range from detection of aspirin and folic acid individually as well as in combination with other compounds or from delivery systems such as lipophilic carriers (Franeta *et al.*, 2002; Kees *et al.*, 1996; Andrisano *et al.*, 2003; Buur *et al.*, 2005). However, to date, there have been no reported protocols using high performance liquid chromatography (HPLC) to simultaneously analyze aspirin and folic acid from polymer nanoparticles. Recent literature postulates that the principal metabolite of aspirin, salicylic acid, is the active moiety that acts in colon cancer chemoprevention (Paterson *et al.*, 2001). Since aspirin itself has a half life of < 30 min (Needs *et al.*, 1985) and is prone to hydrolysis in aqueous surroundings (for example, in body fluids) the HPLC protocol developed for this study determined the active moiety of

aspirin, salicylic acid (Wu, 2005) which was obtained by hydrolyzing the aspirin in presence of two drops of 0.1 N sodium hydroxide solution. Thus, salicylic acid was simultaneously determined along with folic acid using this methodology. Development of this methodology would allow the quantification of trace quantities of the drugs released from nanoparticles thus facilitating a much needed and timely process as more evidence becomes available regarding the combined chemoprevention of colon cancer using these drug combinations. Thus, the objective of this study was to develop a simple HPLC method for the simultaneous determination of aspirin (as salicylic acid) and folic acid (Figs. 1 a,b and 2) released from PLGA 50:50 co-polymer nanoparticles for the chemoprevention of colon cancer.

Experimental

Materials

For the development of nanoparticle formulations, aspirin and polyvinyl alcohol (PVA, used as emulsion stabilizer) were purchased from Spectrum Chemicals (Gardena, CA). Folic acid and dichloromethane (DCM) were obtained from Fisher Scientific (Houston, TX) and the poly-lactide-co-glycolide (PLGA) co-polymer (50:50 grade) was supplied by Durect Corp. (Pelham, AL). For the HPLC method development, HPLC grade acetonitrile solvent were obtained from Fisher Scientific (Houston, TX), and tri-fluoroacetic acid (TFA) from Sigma Chemicals (St. Louis, MO). Distilled water was used for all the experiments.

Equipment and Chromatographic Conditions

The analyses were performed using a Shimadzu LC-20 binary HPLC system (Columbia, MD) consisting of two LC 20-AT dual plunger pump, a SIL-20A automatic sample injector, an SPD – M20A photodiode array (PDA) detector set at 233 nm for aspirin and 277 nm for folic acid, and a CBM-20A “Prominence” controller system. The column used was a Phenomenex Luna C-18(2) (4.6 mm × 150 mm, I.D. 5 µm) (Torrance, CA). The EZ Start v. 7.4 software was used to collect, integrate and analyze the chromatographic data. The mobile phase was a mixture of acetonitrile (ACN) – 0.1% trifluoroacetic acid (TFA) solution used as a 30 min gradient method with the conc. of ACN varying from 12.5 - 100 - 12.5% acetonitrile in 0.1% TFA with the flow rate set at 0.5 mL/min (Table 1).

Preparation of Standard Solutions

Stock solutions of aspirin were prepared by dissolving 10 mg aspirin in 10 ml methanol (1 mg/ml concentration) whereas 10 mg folic acid was dissolved in a 10 ml of distilled water into which 0.1 N sodium hydroxide (NaOH) was added to facilitate dissolution, and to facilitate the hydrolysis of aspirin to salicylic acid and acetic acid (Fig. 2). Caffeine dissolved in distilled water (0.5 mg/ml) was used as the internal standard solution and 10 µl (5 µg quantity) was added to each vial containing the samples. Calibration curves were prepared by diluting the stock solutions to represent final concentration range of 0.1 – 100 µg/ml (0.1, 0.5, 1, 5, 10, 25, 50, 75 and 100 µg/ml).

Preparation of Samples

1 ml aliquot of the unknown aspirin (as salicylic acid) and folic acid in methanol and water respectively were mixed together and spiked with the caffeine internal standard working solution (10 µl) and vortexed for 1 min. 50 µl injections of samples were made into the HPLC system after filtering the samples through a micrometer syringe.

Standard/Calibration curves

The calibration curves consisted of various concentrations of folic acid and aspirin (as salicylic acid) using 10 µl of caffeine as an internal standard. The concentrations of each of

the drugs ranged from 0.1 to 100 µg/ml. The regression equation was calculated by a weighted least-squares linear regression analysis of peak area ratios (salicylic acid or folic acid/I.S.) versus the aspirin (as salicylic acid) and folic acid concentrations.

Method Validation

The method was validated in agreement with the International Conference on Harmonization Guidelines (ICH, 1996) with regards to its specificity, linearity, limits of detection (LOD), limits of quantification (LOQ), accuracy and precision. The LOD was selected at a signal/noise (S/N) ratio of 3. The LOQ was selected at an S/N ratio of 10. The noise was determined by calculating the area under the curve (AUC) of a blank sample. The intra- and inter-day accuracy and precision were evaluated by assaying triplicate samples of blinded concentrations of aspirin and folic acid representing low, middle and high concentrations. The intra-day variation was determined by evaluating triplicate measurements of each blinded sample on one single day whereas the inter-day variation was assayed for triplicate measurements of each blinded sample for three consecutive days with unknown samples being prepared daily. The precision was expressed as the relative standard deviation (RSD). The accuracy was determined by comparing the calculated concentration from the standard curves to the theoretical concentration.

Preparation of novel drug loaded PLGA 50:50 nanoparticle formulations

To encapsulate aspirin and folic acid into PLGA polymer nanoparticles, a water/oil/water (w/o/w) multiple emulsion process of solvent evaporation was used (Florence et al., 1995; Prabhu et al., 2002). At first, 5 ml of 2% PVA aqueous solution (inner water phase) was mixed with 10 ml of dichloromethane (DCM, oil phase) into which 500 mg PLGA 50:50 copolymer was dissolved. The mixture was ultra-sonicated (Branson, Los Angeles, CA) to create the primary (w/o) emulsion, which was subsequently introduced in a drop-wise manner into 40 ml of 2% PVA solution (outer water phase) thus completing the formation of a w/o/w emulsion. The DCM solvent was partially evaporated for 2 h at ambient temperature to create nascent “embryonic” nanoparticles which formed as the PLGA copolymer dissolved within the organic solvent started to precipitate due to evaporation of the organic solvent. At this point, 1 g of the chemopreventive agent, aspirin was added to the prepared w/o/w emulsion. The emulsion was ultra-sonicated for 3 min and again subjected to evaporation of DCM for an additional 3 – 4 h. Thus, the complete evaporation of the organic solvent DCM resulted in precipitation of the drug loaded PLGA 50:50 copolymers in the form of nanoparticles within the remaining aqueous medium. Finally, the nanoparticles were washed with water to remove excess free drugs from the surface, freeze-dried (Labconco, Kansas City, MO) and subjected to particle sizing, encapsulation efficiency and HPLC method application using drug release studies. Folic acid nanoparticles were prepared separately using the same procedure as outlined above. For studies related to application of the HPLC method, fractions of aspirin and folic acid nanoparticles were mixed together. Particle sizing was conducted on all samples using 5 mg of each nanoparticle formulation, dissolved in 10 ml of water and a Nicomp submicron particle sizer (Model-370) was used for size determination.

Application of the Method

Encapsulation efficiency—For encapsulation efficiency, 5 mg of each nanoparticle formulation (aspirin or folic acid) was dissolved in 10 ml DCM to dissolve the PLGA copolymer and release the free drugs, which were subsequently extracted in distilled water and analyzed using the HPLC method. Encapsulation efficiency was determined using the following expression:

$$\text{Amount (mg) per HPLC method/theoretical yield (mg) X100}$$

Drug release studies—Aspirin (as salicylic acid) and folic acid nanoparticles were subjected to *in vitro* controlled drug release studies. Samples, in triplicate, of each nanoparticle formulation (100 mg) was mixed in a 1:1 ratio and suspended in 5 ml of distilled water, incubated at 37°C and sampled at fixed intervals (0.5, 1.5, 4, 16, 21, 26, 38, 46, 72 h). After every sample withdrawal, fresh distilled water was added to replace the sample. All samples were centrifuged at 3000 rpm for 3 min, filtered and subjected to HPLC analysis using the validated method.

Results and Discussion

Method Validation

In this present work, a new reversed-phase HPLC method for simultaneous determination of salicylic acid and folic acid from nano-particulate systems was examined. Fig. 3 a and b shows the typical chromatograms for salicylic acid and folic acid at wavelengths of 233 nm and 277 nm, respectively. The retention time was 21 min for salicylic acid, 8.9 min for folic acid and 11.4 min for caffeine (used as internal standard). The retention times were deliberately extended to get optimum peak resolution. As such, at earlier retention times, there was significant distortion of peaks that were noted for salicylic acid. At the above wavelengths, as detected by the PDA detector, there were no disturbances in the chromatograms such as contaminating peaks, peak splitting or tailing. The absence of interfering peaks indicates that no contamination has occurred due to degradation of the samples due to the rapid analysis method.

Linearity

The linearity of the method was established by calculating the calibration curves determined by the peak area ratio of salicylic acid (Fig. 4 a) or folic acid (Figure 4 b) at different concentrations and the internal standard in the range of 0.1– 100 µg/ml (0.1, 0.5, 1, 5, 10, 25, 50, 75 and 100 µg/ml). Each sample was analyzed in triplicate. For both agents, the linearity of the curves was good in the range evaluated. The equation of the calibration curve and regression coefficient for salicylic acid was:

$$\begin{aligned}\text{Area} &= 0.1354 * C \\ \text{Correlation coefficient (R}^2\text{)} &= 0.9996 \text{ (n=27)}\end{aligned}$$

Whereas, the equation for folic acid was as follows:

$$\begin{aligned}\text{Area} &= 0.1707 * C \\ \text{Correlation coefficient (R}^2\text{)} &= 0.9998 \text{ (n=27)}\end{aligned}$$

The R² obtained for both chemopreventive agents was higher than 0.999 which indicated a good linearity according to the recommendations from the Center for Drug Evaluation and Research (US-FDA) and ICH guidelines (Epshtein, 2004).

Precision

The precision of the method was assessed using samples at three different concentrations (1, 10, 100 µg/ml). As recommended by the ICH, the concentrations selected were within the working range regarding linearity. For the intra-day variation determination three standard solutions were analyzed on the same day. For inter-day variation these samples were analyzed three times on three different days. As shown in Table 2, the intra-day relative standard deviations (RSD) for aspirin ranged from 2.26 – 7.71 whereas that of folic acid ranged from 1.28 – 6.04. Inter-day RSD for aspirin was 1.90 – 5.51 and folic acid ranged from 1.87 – 3.15 for the same concentrations of standard solutions. Thus, the low RSD values for both aspirin and folic acid method demonstrated good repeatability and precision (Pedro *et al.*, 2009; Bresolle *et al.*, 1996).

Accuracy

The obtained experimental results when compared to the actual concentration values gives the accuracy of the methodology. This was assessed by the determination of percentage recovery of known amounts of aspirin and folic acid prepared as standard solutions. As shown in Table 3, three different standard solutions of 1, 10 and 100 µg/ml were prepared and analyzed with a mean recovery of $99.67 \pm 3.47\%$ for aspirin (as salicylic acid) and $100.9 \pm 2.4\%$ for folic acid was found. In compliance with ICH guidelines and recommendations (Epshtein, 2004) the data obtained for both aspirin (as salicylic acid) and folic acid showed close agreement of theoretical to that of measured concentration values. The established acceptance criteria for accuracy ($RSD < 5.0\%$) was met showing that the analytical method is precise and accurate.

Detection Limit and Quantitation Limit

In terms of the sensitivity of the assay, as shown in Table 4, the limit of detection (LOD) for salicylic acid and folic acid was 0.03 µg/ml and 0.01 µg/ml, respectively. The limits of quantification (LOQ) were 0.1 µg/ml for salicylic acid and 0.05 µg/ml for folic acid. Thus, a high sensitivity of the methodology was demonstrated with the ability to quantify trace quantities of both compounds.

Application of the Method

The encapsulation efficiency (%) of aspirin in the PLGA 50:50 copolymer matrix was $87 \pm 9\%$ whereas folic acid demonstrated $70 \pm 15\%$ encapsulation thus indicating that sufficient drug was encapsulated within the polymer matrix and was available for release. Particle sizing studies of drugs encapsulated within the polymer matrix showed a size range of 577 nm for aspirin and 431 nm for folic acid (Table 5). Release of aspirin (as salicylic acid) and folic acid from PLGA 50:50 nanoparticles was measured for 3 days using the validated HPLC method. As shown in Figure 5, the release of salicylic acid was quicker than that of folic acid for the entire duration of the study. A cumulative release of approximately 8 mg of salicylic acid was observed after three days of release from polymer nanoparticles. In comparison, folic acid showed only about 2 mg release over the duration of the study thus representing a 4-fold decrease in release patterns. Thus, the newly developed HPLC methodology allowed the determination of aspirin (as salicylic acid) and folic acid simultaneously from the nano-particulate system.

Previous studies have shown variable levels of sensitivity with the HPLC method of analysis of aspirin and folic acid (McMahon *et al.*, 1998; Breithaupt, 2001). Although no group has ever simultaneously detected aspirin and folic acid from nanoparticulate systems, limited information on individual studies of aspirin and folic acid are available (Sawyer *et al.*, 2003; Amidzic, *et al.*, 2005). Sawyer (2003) reported the range of linearity of aspirin to be within

0.5 – 1.25 mg/mL with overall recovery of the drug at 100.2%. Another study showed linearity of aspirin in the concentration range of 0.1 – 5 µg/mL with a limit of detection and quantification found at 0.04 µg/mL and 0.10 µg/mL, respectively (McMahon, et al., 1998). With folic acid, limits of detection and quantitation have been reported at 0.02 and 0.07 µg/mL (Andrisano et al., 2003) and recovery at approximately 97% in 0.1M acetate buffer (Breithaupt, 2001). In comparison, as demonstrated from our studies, the simultaneous determination of both aspirin and folic acid using a validated HPLC method shows greater sensitivity and reproducibility than other published methods in literature. In addition, we present a simple method which can be replicated with ease.

Conclusion

Thus, the results from this work clearly show the development and validation of a new, rapid RP-HPLC method of analysis of aspirin (as salicylic acid) and folic acid from polymer based nano-particulate systems. The assay has been shown to be precise, accurate and specific with very low detection and quantitation limits thus meeting the recommendations of the ICH. In addition, this method successfully achieved the characterization of drugs aspirin (as salicylic acid) and folic acid being released from nano-particulate systems demonstrating its suitability as a therapeutic agent for future studies in cancer chemoprevention.

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(a) Aspirin

(b) Folic acid

Figure 1.
Chemical structures of aspirin (a) and folic acid (b).

Figure 2.
Aspirin hydrolysis reaction.

Figure 3.

Representative chromatograms of (a) aspirin (as salicylic acid, s.a.) and (b) folic acid (f.a.) detected at 233 nm and 277 nm respectively. The mobile phase was a mixture of acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) solution used as a 30 min gradient method with the conc. of ACN varying from 12.5 - 100 - 12.5% with the flow rate set at 0.5 mL/min.

a. salicylic acid

b. folic acid

Figure 4. Standard calibration curves for (a) aspirin (as salicylic acid), $R^2 = 0.9996$ and (b) folic acid, $R^2 = 0.9998$.

Figure 5. Application of the RP-HPLC method demonstrating the analysis of aspirin and folic acid released from PLGA 50:50 copolymer nano-particulate system.

Table 1

Acetonitrile (ACN) – 0.1% trifluoroacetic acid (TFA) gradient method with the conc. of ACN varying from 12.5 - 100 - 12.5% in 0.1% TFA (flow rate: 0.5 mL/min)

Time (min)	Acetonitrile (%)
0	12.5
2	20
4	30
16	50
22	100
23	12.5
30	12.5

Table 2

Results of precision tests for the determination of aspirin (as salicylic acid) and folic acid from standard solutions.

Compound	Standard solution($\mu\text{g/mL}$)	n	Measured ($\mu\text{g/mL}$)	R.S.D.
Intra-day				
Salicylic acid	1	3	0.94 ± 0.07	7.71
	10	3	9.54 ± 0.34	3.53
	100	3	99.98 ± 2.26	2.26
Folic Acid	1	3	0.97 ± 0.01	1.28
	10	3	10.63 ± 0.64	6.04
	100	3	98.83 ± 1.78	1.80
Inter-day				
Salicylic acid	1	3	1.01 ± 0.03	2.92
	10	3	9.92 ± 0.55	5.51
	100	3	98.81 ± 1.92	1.9
Folic Acid	1	3	1.00 ± 0.03	3.15
	10	3	10.34 ± 0.19	1.87
	100	3	99.30 ± 2.32	2.33

Table 3

Results of recovery (%) for aspirin and folic acid from standard solutions

Compound	Standard solution (µg/mL)	n	Accuracy % (Recovery ± S.D.)%
Salicylic acid	1	3	101 ± 3
	10	3	99.2 ± 5.5
	100	3	98.81 ± 1.92
Folic Acid	1	3	100 ± 3
	10	3	103.4 ± 1.9
	100	3	99.30 ± 2.32

Table 4

Results of the limits of detection (LOD) and quantification (LOQ) for aspirin and folic acid.

Compound	LOD (µg/mL)	LOQ (µg/mL)
Salicylic acid	0.03	0.10
Folic acid	0.01	0.05

Table 5

Particle sizing (nm) and encapsulation efficiency (%) of drug loaded polymer nanoparticles

Compound	Particle size (nm)	Encapsulation Efficiency (%)
Aspirin	577 ± 139	87 ± 9 %
Folic acid	431 ± 184	70 ± 15%