ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
15 (1996) 73–82

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

# Development and validation of a reversed-phase liquid chromatographic method for analysis of aspirin and warfarin in a combination tablet formulation

Eda Ross Montgomery*, Suzanne Taylor, James Segretario, Elizabeth Engler, Dolores Sebastian

*Pharmaceutical Research and Development, The DuPont Merck Pharmaceutical Company, Experimental Station, P.O. Box 80353, Wilmington, DE 19880-0353, USA*

Received for review 2 October 1995; revised manuscript received 2 February 1996

**Abstract**

A stability-indicating liquid chromatographic method for the simultaneous analysis of aspirin and warfarin in warfarin sodium/aspirin combination (DuP 647) tablets has been developed and validated. This paper presents linearity, accuracy, precision, robustness, recovery, limits of detection and quantitation, and cross-validation data. The method has been shown to be specific and stability-indicating, and to give results comparable to existing methods for the individual components. Solution stability has been optimized for routine analysis.

*Keywords:* Aspirin; Pharmaceutical analysis; Reversed-phase liquid chromatography; Stability-indicating; Warfarin

## 1. Introduction

The combination of warfarin sodium and aspirin (DuP 647) is in Phase III clinical studies for the potential treatment of post-myocardial infarction. Two strengths of tablets are under development: 3 mg warfarin sodium with 80 mg aspirin (3/80 mg tablets) and 1 mg warfarin sodium with 80 mg aspirin (1/80 mg tablets). The large difference in concentration, solubility differences, and the interactions between the active ingredients have presented some unique challenges to the development and validation of suitable methods for routine analysis. Although separate stability-indicating methods exist for both aspirin and warfarin [1–5], none of these methods is capable of simultaneously analyzing both active ingredients.

This paper describes the development and validation of a stability-indicating method which is capable of simultaneously analyzing both aspirin and warfarin in the combination tablets. Data supporting the linearity, accuracy, precision, ruggedness, specificity, and limits of detection and quantitation for potential degradation products are presented. In addition, results generated using these methods were compared to results generated

* Corresponding author. Tel.: (+1) 302-695-1639; fax: (+1) 302-695-3705.


*PII* S0731-7085(96)01813-4

from methods which separately analyze aspirin and warfarin and their potential degradation products.

## 2. Experimental

### 2.1. Equipment

Chromatographic results were obtained using five chromatographic systems. The first consisted of a Hewlett Packard 1090L instrument and ICOS automated methods development software (Hewlett Packard, Palo Alto, CA). The second system was a Hewlett Packard 1050 Series pump, autosampler, column oven, and detector. The third system consisted of a Spectra Physics Analytical Spectra System P4000 pump (Spectra Physics Analytical, San Jose, CA), Alcott 728 autosampler (Micromeritics Corp., Norwood, GA), Waters column oven (Millipore Corp., Milford, MA), and an ABI Spectroflow 783 UV detector (Applied Biosystems International, Foster City, CA). The fourth system was a Spectra Physics Analytical SpectraSystem Series 8500 Pump, WISP 712 autosampler (Millipore Corp.), Waters column oven (Millipore), and an ABI Spectroflow 757 UV detector (Applied Biosystems International). The fifth system consisted of Waters 510 pumps (Millipore), WISP 712 autosampler (Millipore Corp.), Waters column oven (Millipore), and an ABI Spectroflow 757 UV detector (Applied Biosystems International).

### 2.2. Materials

For all validation experiments, the warfarin and aspirin standards used were Warfarin (free acid) Reference Standard (in-house standard) and Aspirin USP Reference Standard. Free salicylic acid was also USP Reference Standard. 4-Hydroxycoumarin, *trans*-4-phenyl-3-buten-2-one, and citric acid were obtained from Aldrich (Milwaukee, WI). Butylparaben internal standard and acetylsalicylic anhydride were obtained from Fluka (Ronkonkoma, NY). Salicylsalicylic acid was obtained from Pfaltz and Bauer (Waterbury, CT). Acetylsalicylsalicylic acid, Alice's ketone (3-(*o*-hydroxyphenyl)-5-phenyl-2-cyclohexen-1-one) [6], and warfarin acetate were prepared in-house and structures were verified by NMR. Structures for all standards and potential related substances are provided in Fig. 1. Acetonitrile, chloroform, and methanol were HPLC grade, obtained from EM Science (Gibbstown, NJ). Distilled, deionized water, obtained from a Milli-Q water purification system (Millipore) was used for the preparation of pH 2.6 water. Formic acid [Kodak (Rochester, NY) or EM Science] was used to adjust pH. All chemicals were used without further purification.

### 2.3. Method development: chromatography

Preliminary isocratic HPLC methods were developed using ICOS automated methods development software and solutions of the active ingredients and all the potential known degradants. It became apparent that an isocratic method would not be suitable due to the large range of polarities of the analytes. Therefore a gradient method, which combined the best isocratic methods, was chosen. This first gradient was the same for both assay and degradation products analysis.

During the course of stability testing, some late-eluting, low-level components appeared in the degradation product analysis. In order to quantitate these components should the need arise, the gradient for degradation products analysis was changed.

### 2.4. Method development: sample preparations

It is well known that aspirin undergoes solvolysis in aqueous solutions or in the presence of alcohols [7]. Accordingly, a single nonaqueous sample-preparation solvent was developed that could be used for warfarin sodium, aspirin, and salicylic acid analysis. Although this sample solvent maximized solution stability, it was not effective in disintegrating the tablets. Therefore, a homogenization procedure was developed to physically break up the tablets. This procedure was included in all of the LC methods. Because of volume limitations of the homogenizer, the concentration of the content uniformity samples and

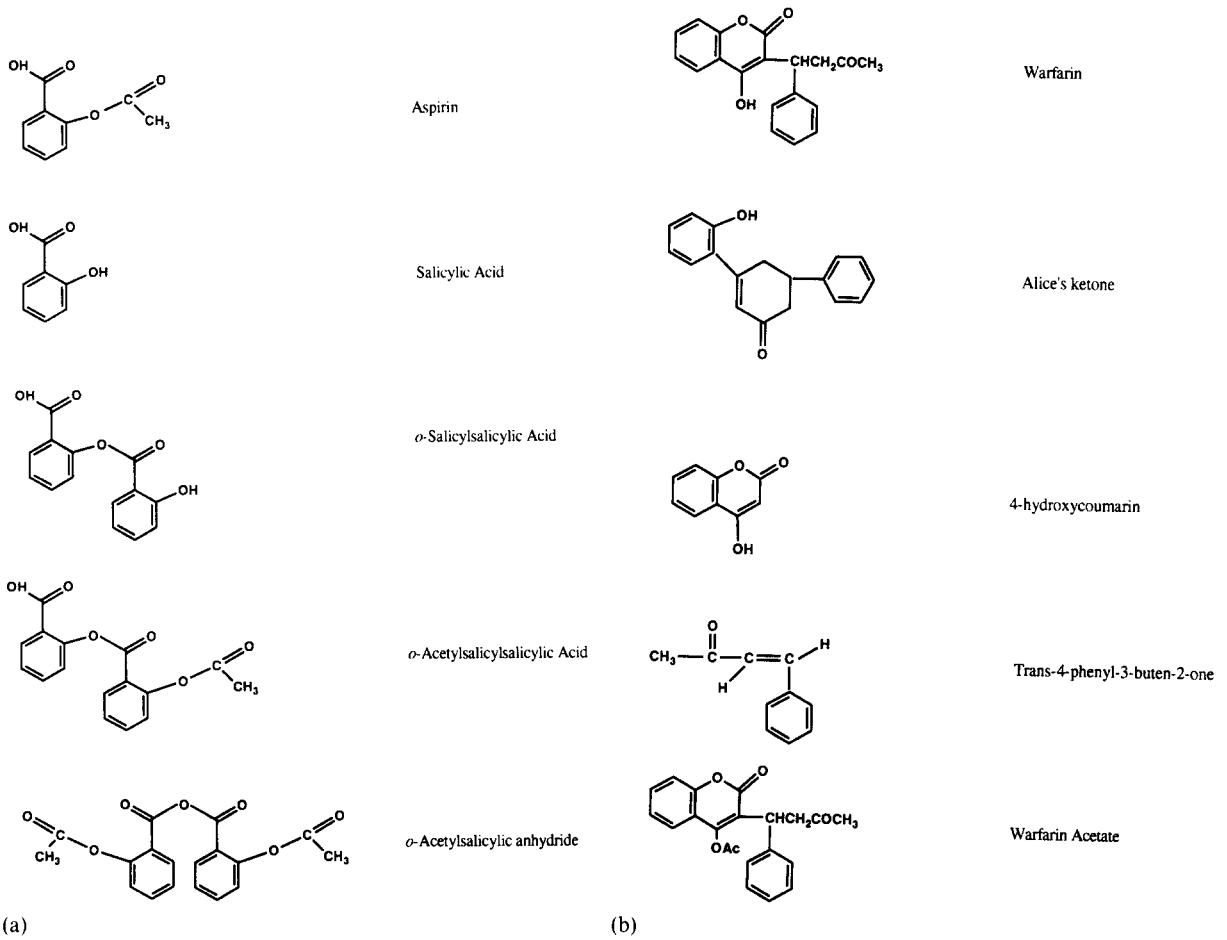


Fig. 1. Structures of (a) Aspirin, (b) Warfarin Sodium and their Potential Related Substances.

standards was halved. The concentration ranges of aspirin and warfarin sodium for the linearity and recovery experiments were broadened to cover both the content uniformity and assay conditions. An internal standard (butylparaben) was also added to the sample solvent for the assay and content uniformity analyses, since the homogenizer did not allow the use of volumetric glassware.

The sample solvent was developed to maximize stability for all components, including minimizing the formation of salicylic acid (a known aspirin degradant [7]). However, even under optimum conditions, salicylic acid levels in the samples increased by approximately 10% over 12 h. This solution stability was unacceptable, given the long run time required for the combined analysis, so a shorter analysis (specific for salicylic acid) was typically used. This method was derived from the USP method for salicylic acid [8] and will not be further discussed.

Since the amount of aspirin in the tablets is fixed (80 mg) while the amount of warfarin varies (1 or 3 mg), the sample concentration for the degradation product analysis was optimized to maximize sensitivity with respect to warfarin degradants. Where necessary, the linearity and recovery ranges were adjusted to span the concentrations of aspirin degradants which resulted.

### 2.5. Chromatographic conitions

Mobile phase A was water, adjusted to pH 2.6 with formic acid. Mobile phase B was methanol,

Table 1
Conditions for the three types of chromatographic analysis (assay, content uniformity, and degradation products)

| Analysis | Gradient profile | Sample solvent | Sample concentration | |
|---|---|---|---|---|
| | | | 1/80 mg strength | 3/80 strength |
| Assay | (A:B:C) (68:17:15 v/v/v) for 11 min, then a linear gradient to (56:17:27, v/v/v) at 15 min. Hold for 38 min, return in 0.5 min to (68:17:15) and equilibrate for at least 6 min before the next injection | Acetonitrile:chloroform:formic acid:citric acid:butylparaben (49.95:49.95:0.1:0.1:0.05, v/v/v/w/w) | 0.04 mg $ml^{-1}$ warfarin, 3.2 mg $ml^{-1}$ aspirin | 0.12 mg $ml^{-1}$ warfarin, 3.2 mg $ml^{-1}$ aspirin |
| Content uniformity | Same as assay | Same as assay | 0.02 mg $ml^{-1}$ warfarin, 1.6 mg $ml^{-1}$ aspirin | 0.06 mg $ml^{-1}$ warfarin, 1.6 mg $ml^{-1}$ aspirin |
| Degradation products | (A:B:C) (68:17:15, v/v/v) for 11 min, then a linear gradient to (56:17:27, v/v/v) at 15 min. Hold for 28 min, then a linear gradient to (13:17:70, v/v/v) at 60 min. Hold for 14 min, return in 1.0 min to (68:17:15) and equilibrate for 7 min before the next injection | Acetonitrile:formic acid:citric acid (99.9:0.1:0.1, v/v/w) | 0.60 mg $ml^{-1}$ warfarin, 48 mg $ml^{-1}$ aspirin | 0.60 mg $ml^{-1}$ warfarin, 16 mg $ml^{-1}$ aspirin |

and mobile phase C was acetonitrile. The mobile phases were filtered, degassed, and pumped at a flow rate of 1.0 ml $min^{-1}$. Injection volume was 20 $\mu$l for all standards and samples. The column used was a Zorbax® C8 (5 $\mu$m, 250 mm × 4.0 mm) from Mac Mod (Chadds Ford, PA). Column temperature was maintained at 40°C. The detector wavelength was 280 nm. The gradient profile, sample solvent, and sample concentration varied with the analysis. These parameters are summarized in more detail in Table 1.

### 2.6. Methods

Linearity was evaluated by preparing standards of warfarin and aspirin which covered all assay and content uniformity concentrations for each component and each strength. Linearity for Alice's ketone was evaluated from 0.044% ($2.67 \times 10^{-4}$ mg $ml^{-1}$) to 0.6% ($3.90 \times 10^{-3}$ mg $ml^{-1}$) relative to warfarin sodium, and linearity for salcylsalicylic acid was evaluated from 0.075% relative to the 3/80 mg tablets (0.012 mg $ml^{-1}$) to 1.27% w/w relative to the 1/80 mg tablets (0.61 mg $ml^{-1}$). The amounts of Alice's ketone and salicylsalicylic acid are quantitated relative to warfarin and aspirin standards respectively, by comparing relative peak areas and correcting for differences in absorptivity using relative response factors for the known related substances. Therefore, the linearity of warfarin was evaluated from 0.03% (0.00019 mg $ml^{-1}$) to 0.60% (0.0036 mg $ml^{-1}$) and linearity of aspirin was evaluated from 0.02% relative to the 3/80 tablets (0.0032 mg $ml^{-1}$) to 1.0% relative to the 1/80 mg tablets (0.48 mg $ml^{-1}$). Unknown degradants are assumed to be degradation products of warfarin with absorptivity equal to that of warfarin.

The limits of detection for aspirin, warfarin, Alice's ketone, and salicylsalicylic acid were determined by diluting solutions of known concentration until the responses were 3–5 times the noise. The limits of quantitation of known aspirin and warfarin degradants in the combination tablets

(Alice's ketone and salicylsalicylic acid) were determined by spiking them into duplicate samples of the tablets at both their limits of detection and quantitation. The limits of quantitation of suspected aspirin degradants (acetylsalicylsalicylic acid and acetylsalicylic anhydride) [7] were determined by spiking duplicate tablet samples at their limit of quantitation. Recovery studies were done at the assay concentrations by spiking warfarin and aspirin in triplicate into placebo tablets at 75%, 100%, and 125% of the label claim.

Solution stability for aspirin and warfarin sodium assay, content uniformity, and degradation products analyses was evaluated by monitoring the response (area or ratio) of the high standards and the chromatographic profile of a sample preparation over at least 72 h at 3 h intervals.

Specificity studies were done by dissolving tablets in both aqueous 0.1 N HCl and $10^{-4}$ N NaOH and monitoring degradation (measured by the decrease in aspirin peak size) as a function of time. The tablets were also subjected to thermal, photochemical, and humidity conditions by storage for 2 weeks at 60°C, 600 foot candle light, and 40°C/75% relative humidity. After degradation, the purities of the aspirin and warfarin peaks were evaluated using diode array UV spectroscopy with a peak purity evaluation program supplied by Hewlett Packard.

Repeatability studies were done by assaying six sample preparations of a single lot of tablets for aspirin and warfarin sodium. Assay method reproducibility and ruggedness were evaluated by comparing the results obtained from multiple sample preparations of a single lot of 1/80 mg tablets on three days by two analysts.

The response factors for potential aspirin and warfarin degradants were measured using the degradation product test conditions. Response factors were determined from injections at the 0.1% level for degradants of both aspirin and warfarin. The response factors for aspirin degradants were calculated relative to aspirin, while those for warfarin degradants were calculated relative to warfarin.

To measure assay and degradation product method robustness, samples and standards were chromatographed using mobile phase with pH values which ranged from 2.3 to 3.1 around the nominal pH value of 2.6.

The combined assay method was compared with the existing methods for aspirin [9] and warfarin [5] analysis by analyzing duplicate sample preparations by each method.

## 3. Results and discussion

### 3.1. Linearity

The linearity of the method for aspirin and warfarin sodium assay and content uniformity was evaluated for aspirin concentrations between 0.77 and 4.65 mg $ml^{-1}$ and warfarin concentrations between 0.01 and 0.18 mg $ml^{-1}$. Linearity of the method for degradation product analysis was evaluated for aspirin concentrations from 0.0032–0.48 mg $ml^{-1}$ and for warfarin concentrations from $1.9 \times 10^{-4}$–$3.6 \times 10^{-4}$ mg $ml^{-1}$. Alice's ketone was evaluated at concentrations from $2.67 \times 10^{-4}$–$3.90 \times 10^{-3}$ mg $ml^{-1}$ and salicylsalicylic acid was evaluated at concentrations from 0.012–0.61 mg $ml^{-1}$. A summary of the data appears in Table 2.

The correlation coefficients for assay of warfarin sodium, aspirin, Alice's ketone, salicylsalicylic acid, and aspirin and warfarin at degradation products levels were all greater than 0.999, with near-zero $y$ intercepts relative to the mid or 0.1% standards respectively. The linearity-determination experiments for warfarin were done separately for each tablet strength; slopes ranged from 3.65–3.79 ml $mg^{-1}$ and intercepts ranged from $-6.5 \times 10^{-3}$ – $-6.7 \times 10^{-3}$. The slopes for the aspirin assay and content uniformity analyses were 0.530 ml $mg^{-1}$ and 0.565 ml $mg^{-1}$ respectively, while the intercepts were $3.7 \times 10^{-2}$ and $-1.4 \times 10^{-3}$ respectively. For both aspirin and warfarin, slopes and intercepts throughout these concentration ranges were similar to each other. For the degradation product analysis conditions, the aspirin slope was $5.35 \times 10^{6}$ $\mu$V s ml $mg^{-1}$ with an intercept of $7.61 \times 10^{3}$ $\mu$V s, while the warfarin slope was $3.62 \times 10^{7}$ $\mu$V s ml $mg^{-1}$ with an intercept of 347 $\mu$V s. Based on these results,

Table 2
Linearity data for aspirin and warfarin for assay, content uniformity, and degradation product analyses

| Component and tablet strength | Type of analysis | Slope (ml $mg^{-1}$) | Y intercept | Correlation coefficient[a] |
|---|---|---|---|---|
| Aspirin (all) | Assay | 0.530 | 0.037 | 0.9996 |
| Aspirin (all) | Content uniformity | 0.565 | −0.014 | 0.9992 |
| Warfarin (3/80 mg) | Assay | 3.65 | −0.0067 | 0.9997 |
| Warfarin (3/80 mg) | Content uniformity | 3.68 | −0.0067 | 0.9997 |
| Warfarin (1/80 mg) | Assay and content uniformity | 3.79 | −0.0065 | 0.9994 |
| Aspirin (all) | Degradation products | $5.35 \times 10^{6}$[b] | $7.61 \times 10^{3}$[b] | 0.9999 |
| Warfarin (all) | Degradation products | $3.62 \times 10^{7}$[b] | $3.47 \times 10^{2}$[b] | 0.9996 |
| Alice's ketone (all) | Degradation products | $1.711 \times 10^{4}$[b] | $-1.171 \times 10^{3}$[b] | 0.9997 |
| Salicylsalicylic acid (all) | Degradation products | $4.544 \times 10^{6}$[b] | 59.18[b] | 1.0000 |

[a] Each regression was done with a minimum of seven points, two injections per point.
[b] Data are based on peak areas (in $\mu$V s). Units are $\mu$V s ml $mg^{-1}$ for slopes, and $\mu$V s for $y$ intercepts.

the methods for assay, content uniformity, and degradation product analysis are linear throughout their working ranges. Since the responses of Alice's ketone and salicylsalicylic acid were also linear throughout their expected ranges, it was possible to quantitate them relative to aspirin and warfarin using a response factor to correct for differences in absorbtivity. Assay and content uniformity analyses are presently performed using a three-point calibration curve. However, since all methods have near-zero intercepts, a single-point calibration is expected to yield equivalent results.

### 3.2. Precision

#### 3.2.1. Repeatability

For six replicate sample preparations, the aspirin results ranged from 106.1% to 111.8% of the label claim, with a mean of 108.3% and 1.7% RSD. Warfarin results ranged from 101.4% to 103.6% of the label claim, with a mean of 102.6% and 0.7% RSD. This is a measure of the method variability that can be expected for a given analyst performing the analysis.

#### 3.2.2. Reproducibility

Reproducibility of the assay method was evaluated from multiple sample preparations of a single lot on three separate days by two analysts, and using a different lot of tablets from that used in the repeatability experiments. The results are shown in Table 3. The grand mean for a total of seven analyses was 103.4% for aspirin (1.5% RSD) and 99.6% for warfarin sodium (1.2%

Table 3
Assay method reproducibility

| Analyst | Day | Aspirin (% of label) | Warfarin (% of label) |
|---|---|---|---|
| 1 | 1 | 102.9 | 99.4 |
| | | 102.6 | 100.3 |
| | | 102.6 | 99.3 |
| Mean | (Analyst 1) | 102.7 | 99.7 |
| % RSD | | 0.2 | 0.6 |
| 2 | 2 | 106.5 | 99.0 |
| | | 102.8 | 102.0 |
| 2 | 3 | 104.4 | 98.6 |
| | | 102.3 | 98.8 |
| Mean | (Analyst 2) | 104.0 | 99.6 |
| % RSD | | 1.8 | 1.6 |
| Grand mean ($n = 7$) | | 103.4 | 99.6 |
| % RSD | | 1.5 | 1.2 |

RSD). The %RSD for aspirin is comparable to that of the repeatability experiments. The %RSD of 1.2% obtained for warfarin, while greater than that of the repeatability experiments, is probably a more realistic measurement of the method precision.

### 3.3. Limits of detection and quantitation

The limits of detection for warfarin and aspirin in the degradation products analysis were 0.03% and 0.004% respectively, measured relative to the components in the 3/80 mg tablets. The limit of detection for Alice's ketone was 0.044%. The limit of detection for salicylsalicylic acid was 0.002% for the 1/80 mg tablets and 0.005% for the 3/80 mg tablets. Detection at these levels was more than adequate for monitoring tablet stability. The limit of quantitation for both aspirin and warfarin degradants was 0.10%.

The limits of detection and quantitation for Alice's ketone and salicylsalicylic acid were verified by spiking the tablets with duplicate preparations of Alice's ketone and salicylsalicylic acid. Recovery of salicylsalicylic acid at the limit of detection was 103.1% with a 1.2% RSD. Recoveries of salicylsalicylic acid and Alice's ketone at the limit of quantitation were comparable: 98.5% with a 1.4% RSD and 97.3% with a 1.5% RSD respectively. These results indicate that Alice's ketone and salicylsalicylic acid can be measured at the limit of quantitation and salicylic acid can be measured at the limit of detection with acceptable accuracy. However, the recovery of Alice's ketone at the limit of detection was 322% with a 3.5% RSD. The accuracy of determination of Alice's ketone below the limit of quantitation decreases because the peak eluted after warfarin and acetylsalicylic anhydride (another potential aspirin degradant). This resulted in difficulty in both detecting and integrating the Alice's ketone peak.

The recovery of two other potential aspirin degradants was determined in duplicate at the limit of quantitation. Acetylsalicylsalicylic acid and acetylsalicylic anhydride have been detected at low levels in stability samples under stress conditions. Recovery of acetylsalicylsalicylic acid was 99.0% with a 1.4% RSD. Recovery of acetylsalicylic anhydride was lower: 82.4% with a 2.3% RSD. Difficulties in detecting and integrating this peak, which appears on the tail of the warfarin peak, probably caused the low recovery. However, both recoveries are acceptable to quantitate the low levels of these potential degradants when system suitability has been met.

Table 4
Recovery of aspirin and warfarin sodium from spiked placebo tablets at 3/80 mg assay concentrations

| % Label | Aspirin (%) | Warfarin sodium (%) |
|---|---|---|
| 75 | 101.0 | 96.2 |
| | 100.9 | 100.2 |
| | 101.0 | 99.9 |
| 100 | 101.1 | 100.7 |
| | 101.2 | 100.5 |
| | 100.4 | 99.0 |
| 125 | 100.2 | 99.4 |
| | 100.2 | 99.4 |
| | 100.3 | 99.3 |
| Mean | 100.7 | 99.4 |
| % RSD | 0.4 | 1.3 |

### 3.4. Recovery

The accuracy of the assay and content uniformity methods was assessed by fortifying placebo tablets with known amounts of aspirin and warfarin at 75%, 100%, and 125% of the highest (3/80 mg assay) and lowest (1/80 mg content uniformity) sample solution concentrations. Mean recoveries (listed in Tables 4 and 5) were 100.7% ± 0.4% RSD and 100.1% ± 0.1% RSD for aspirin, and 99.4% ± 1.3% RSD and 99.8% ± 0.5% RSD for warfarin sodium. Since the recovery for both sets of conditions was within one standard deviation of 100%, the method is capable of accurately quantitating aspirin and warfarin sodium at these concentrations.

### 3.5. Solution stability

Solution stability was evaluated for standards and samples for the assay, content uniformity,

Table 5
Recovery of aspirin and warfarin sodium from spiked placebo tablets at 1/80 mg content uniformity concentrations

| % Label | Aspirin (%) | Warfarin sodium (%) |
|---|---|---|
| 75 | 100.3 | 99.8 |
| | 100.1 | 99.2 |
| | 100.2 | 100.0 |
| 100 | 100.1 | 100.4 |
| | 99.8 | 100.4 |
| | 100.0 | 99.2 |
| 125 | 100.1 | 99.2 |
| | 100.1 | 99.6 |
| | 100.1 | 100.0 |
| Mean | 100.1 | 99.8 |
| % RSD | 0.1 | 0.5 |

and degradation product analyses. A summary of the solution stability data appears in Table 6. For assay and content uniformity, peak ratios for standards and samples did not vary significantly from the initial response over 72 h. In addition, the standards for the degradation products analysis were stable for 1 week after preparation. The stability of the degradation products samples was evaluated by looking for new degradation products as a function of time because no quantifiable degradants appeared in the samples. Based on this evaluation, degradation products samples were stable for 72 h after preparation.

### 3.6. Cross-correlation of assay data

Data generated by existing methods for aspirin [9] and warfarin sodium tablets [5] were compared to data generated by the combined method. The results for aspirin using the separate method were 108.6% of the label claim, with a 2.8% RSD. The results using the combined method were more precise: 105.6% with a 1.6% RSD. The percentage of the label claim for warfarin sodium was 100.9% $\pm$ 0.5% RSD using the separate method,

Table 6
Summary of solution stability data

| Component | Analysis | Initial ratio of analyte to internal standard | Final ratio of analyte to internal standard | % RSD throughout the run |
|---|---|---|---|---|
| Aspirin high standard | 3/80 mg assay | 2.02118 | 2.05221 | 0.47 |
| Warfarin high standard | 3/80 mg assay | 0.52148 | 0.53465 | 0.70 |
| Aspirin sample | 3/80 mg assay | 1.85788 | 1.89055 | 0.40 |
| Warfarin sample | 3/80 mg assay | 0.40547 | 0.41670 | 0.60 |
| Aspirin high standard | 3/80 mg content uniformity | 1.04562 | 1.03977 | 0.24 |
| Warfarin high standard | 3/80 mg content uniformity | 0.27979 | 0.28162 | 0.29 |
| Aspirin sample | 3/80 mg content uniformity | 1.00318 | 1.00456 | 0.21 |
| Warfarin sample | 3/80 mg content uniformity | 0.20837 | 0.20936 | 0.52 |
| Component | Analysis | Intitial area[a] | Final area[a] | % RSD throughout the run |
| Aspirin high standard | Degradation products | 666015 | 681058 | 0.8 |
| Warfarin high standard | Degradation products | 57562 | 58755 | 1.6 |

[a] Area is in $\mu$V s.

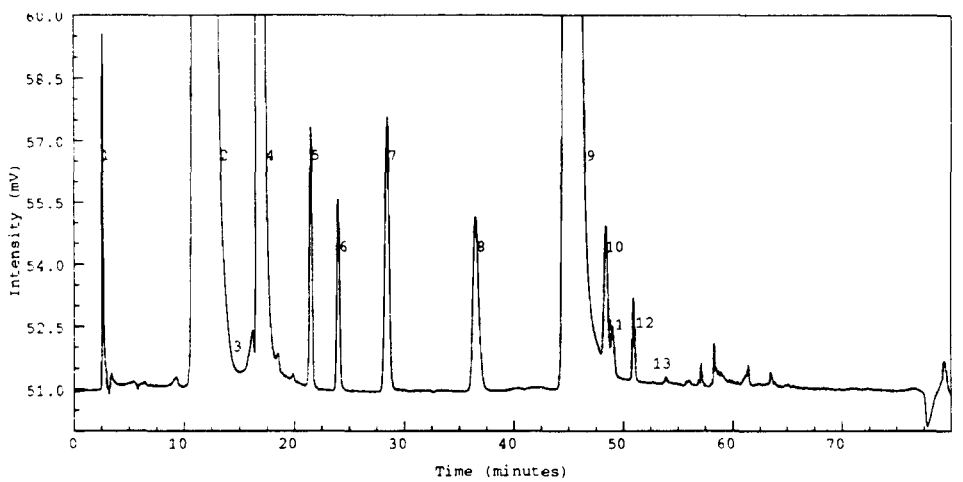


Fig. 2. Chromatogram of Aspirin and Warfarin Spiked with Their Potential Related Substances. Peak identities: (1) solvent, (2) aspirin, (3) 4-hydroxycoumarin, (4) salicylic acid, (5) solution degradant of aspirin (does not appear in fresh samples), (6) *trans*-4-phenyl-3-buten-3-one, (7) acetylsalicylsalicylic acid, (8) salicylsalicylic acid, (9) warfarin, (10) acetylsalicylic anhydride, (11) Alice's ketone, (12) warfarin acetate, (13 and beyond) gradient peaks. Analysis was done using the degradation product gradient listed in Table 1.

and $100.1\% \pm 0.8\%$ RSD from the combined method. The data show that the combined method gives equivalent results to the separate methods.

### 3.7. Specificity

Neither the formulation ingredients nor potential degradants interfere with quantitation of aspirin and warfarin. Fig. 2 is a chromatogram of aspirin and warfarin spiked with their known potential related substances.

To ensure that the method was stability-indicating and specific for both aspirin and warfarin and to check for interactive degradation products, stress studies to force degradation were performed on the combination tablets. No stress studies of aspirin or warfarin sodium alone were deemed necessary since their degradation pathways are well known [6,7]. The tablets were dissolved in 0.1 N HCl and $10^{-4}$ N NaOH and held at room temperature for approximately 16 h. These conditions caused the peak area of the aspirin in the samples to decrease by 5–15%, indicating degradation. After completion of the stress studies, portions of both samples were spiked with 0.5% of known warfarin-sodium-related substances to verify specificity for both the aspirin and the potential warfarin degradants. Separate samples were also subjected to thermal, photochemical, and humidity stress conditions.

All samples (including those subjected to thermal, photochemical, and humidity stress conditions) were analyzed using the aspirin and warfarin sodium assay chromatographic conditions. No evidence of interactive degradation products was seen during the evaluation. However, during stability studies, low levels of warfarin acetate ($\approx 0.05\%$) have been detected. The nature of this degradation pathway will be further investigated as development of the combination tablets moves forward.

In addition to spiking experiments to determine assay specificity, peak purity was evaluated using a diode-array UV detector and peak purity software which compared spectra at the peak upslope, apex and downslope. The spectra were normalized and superimposed for comparison. If identical spectra are found for each point on the peak, then the peak is considered pure. This purity can be measured numerically by comparing the match value for the sample to the average match of five replicate injections of the reference standard. If the sample match is less than three deviations

from the mean of the reference standard match, the peak is considered pure.

Using this approach, the aspirin peak in all samples was determined to be pure. For the concentrations at which aspirin's purity was measurable, the warfarin peak was too small for the same evaluation. Attempts to increase the concentration were unsuccessful, due to solubility limitations of warfarin sodium. However, no evidence of warfarin degradation or changes in its diode array spectrum were seen in any of the stressed samples or in stability studies.

### *3.8. Response factors*

The response factors for potential degradants are the ratios of the areas from injections of known concentrations of aspirin and warfarin to the areas of injections of known concentrations of each related substance. The response factors were: 1.0 for Alice's ketone (relative to warfarin), 0.63 for salicylsalicylic acid, 0.64 for acetylsalicylsalicylic acid, and 0.72 for acetylsalicylic anhydride (all relative to aspirin). Use of these response factors allows these compounds to be quantitated versus the aspirin or warfarin standards in the degradation product method.

### *3.9. Robustness*

The robustness of the method with respect to mobile phase pH was evaluated by determining assay results and evaluating degradation product chromatography (tailing) on the same sample at pH 2.3, 2.6, 2.8, and 3.1. Aspirin assay results in order of increasing pH were 103.7% ± 2.0% RSD, 104.2% ± 1.2% RSD, 104.1% ± 1.5% RSD, and 104.4% ± 1.6% RSD. Warfarin assay results in order of increasing pH were 98.9% ± 1.6% RSD, 99.5% ± 1.0% RSD, 99.7% ± 0.8% RSD, and 99.8% ± 1.6% RSD. In order to obtain optimal precision (particularly for the warfarin sodium assay), the pH should be kept between 2.6 and 2.8. Likewise, for the degradation product analysis, the peak shapes showed significant tailing at the pH extremes (2.3 and 3.1). Therefore, the pH of the aqueous portion of the mobile phase should also be maintained between 2.6 and 2.8 for degradation product analysis.

## 4. Conclusions

The method's linearity, precision and recovery are excellent. Degradation products can be determined with acceptable precision at the limits of quantitation. Further, the method has been shown to be specific, stability-indicating, and to give results comparable to existing methods for the individual components. Solution stability has been optimized for routine analysis. Use of the combined method is more efficient than analysis of aspirin and warfarin sodium using separate methods.

## Acknowledgements

The authors thank Drs. Jim Shea and Frank Diana for helpful advice and technical discussions.

## References

[1] F.E. Blondino and P.R. Byron, J. Pharm. Biomed. Anal., 13 (1995) 111–119.
[2] G. Santoni, L. Fabbri, P. Gratteri, G. Renzi and S. Pinzauti, Int. J. Pharm., 80 (1992) 263–266.
[3] S. Torrado, S. Torrado and R. Cadorniga, J. Pharm. Biomed. Anal., 12 (1994) 383–387.
[4] R.N. Galante, J.C. Egoville, A.J. Visalli and D.M. Patel, J. Pharm. Biomed. Anal., 70 (1981) 167–169.
[5] United States Pharmacopeia, 23rd ed., Rand McNally, Taunton, MA, 1994.
[6] A.O. Robertson, Ph.D. Thesis, University of Wisconsin, Madison, WI, 1953.
[7] C.A. Kelly, J. Pharm. Sci., 59 (1970) 1053–1079.
[8] Ref. [5], pp. 134, 135.
[9] Ref. [5], pp. 1132–1134.