ORIGINAL ARTICLE

DEVELOPMENT, VALIDATION AND KINETIC APPLICATION OF SPECTROMETRIC METHODS FOR THE ANALYSIS OF ASPIRIN, SALICYLIC ACID, AND CAFFEINE AS SINGLE-COMPONENT AND IN MIXTURES

Aqeela Khurshid[1], Zubair Anwar[2*], Adeela Khurshid[1], Iqbal Ahmad[2]
[1]Faculty of Pharmacy, Jinnah University for Women, Karachi, Pakistan
[2]Baqai Institute of Pharmaceutical Sciences, Baqai Medical University, Karachi, Pakistan
Received: November 23, 2017 Accepted: February 20, 2018

ABSTRACT
Aspirin (ASA) and aspirin-caffeine dosage forms are widely used as analgesic drugs. In the present study specific stability-indicating two- and three-component spectrometric methods have been developed for the assay of ASA and its degradation product, salicylic acid, in the presence and absence of caffeine (CF). The methods have been validated with respect to different parameters such as linearity, range, accuracy, precision, reproducibility, limit of detection, limit of quantification and specificity. The results indicated that the proposed methods are linear in the range of 0.0005–0.005 mg%, highly accurate (99.0–100.1%), precise (<3.0% RSD) and robust (<2.0% RSD). These methods have been applied for the assay of ASA in its thermally degraded solution and in degraded tablets. The apparent first-order rate constants (k_{obs}) for the degradation of ASA at pH 8.0–10.0 in the absence and presence of CF are in the range of 5.37–11.0×10^{-3} min^{-1} and 4.99–10.30×10^{-3} min^{-1}, respectively. The accuracy and specificity of the methods have been confirmed from the linearity of first-order plots around the assay data for the degradation of ASA. These methods can accurately determine ASA in the presence of interfering substances.

Keywords: Aspirin, caffeine, kinetics, multicomponent-spectrometric stability-indicating method, salicylic acid.

1. INTRODUCTION
Aspirin (ASA), which is also termed as acetylsalicylic acid (1) (Fig. 1), was synthesized by Felix Hofmann in 1899. It is an analgesic and is also used in the treatment of patients having acute coronary syndromes and also in ischemic stroke[1]. It is effective against the primary and secondary prevention of myocardial infarction, stroke, and cardiovascular death and also used in the management of acute myocardial infarction, unstable angina, and embolic stroke[2]. ASA is believed to be effective in the prevention of acute coronary syndrome, stable angina, revascularization, stroke, and arterial fibrillation[3]. A number of workers have employed various techniques for the analysis of ASA which include UV/Vis spectrometry[4-14], mass spectrometry[15-17], fluorimetery[18-24], chromatography[25-39], electrophoresis[40-44] and electrochemical methods[45-49].

Salicylic acid (SA) (2) (Fig. 1) is a monohydroxybenzoic acid which is a phenolic acid or a beta-hydroxyl acid. In 1826 a German chemist, Johann Andreas Buchner, isolated an extract from the bark of white willow and called it salicin. This salicin was converted into sugar which was then oxidized to SA[50]. It is an anti-inflammatory drug used in lowering the fever and also for the treatment of aches and pains[51]. Some researchers also considered salicylate as a micronutrient in humans and termed it as vitamin S[52]. SA with other beta-hydroxyl acids is the main product in the formulations that are used in the treatment of seborrhea dermatitis, acne, psoriasis, calluses, corns, keratosis pilaris and warts[53]. Different techniques have been used for the analysis of SA which includes UV/Vis spectrometry[6,54-56], fluorimetry[57], liquid chromatography (LC)[29], high-performance liquid chromatography (HPLC)[58-65], gas-liquid

* Corresponding Author Email: zubair_ana@hotmail.com

chromatography (GLC)[6,67,] and voltammetry[68]. Caffeine (CF) (3) (Fig. 1) was first isolated in 1820 by a German chemist Friedlieb Ferdinand Runge from coffee. It has a bitter taste and it is a white crystalline xanthine alkaloid which acts as a stimulant drug and also acts as an inhibitor of acetylcholinesterase. CF is a central nervous system (CNS) and metabolic stimulant[69] and it is used to reduce physical fatigue and to restore alertness when drowsiness occurs[70]. It also affects the sleep deprivation but it may lead to subsequent insomnia[71]. In athletics, the moderate doses of CF can be helpful to improve the sprint[72] whereas its high doses can impair the performance of athletes[73]. It is used in the treatment of a breathing disorder (apnea of prematurity) and is also helpful in the prevention of bronchopulmonary dysplasia in premature infants[74]. Various analytical techniques have been used to analyze CF which include UV/Vis spectrometry[75-82], mass spectrometry[83,84], Fourier transform infrared (FTIR) spectrometry[85-89], nuclear magnetic resonance (NMR)[90], HPLC[91-93] and voltammetry[94].

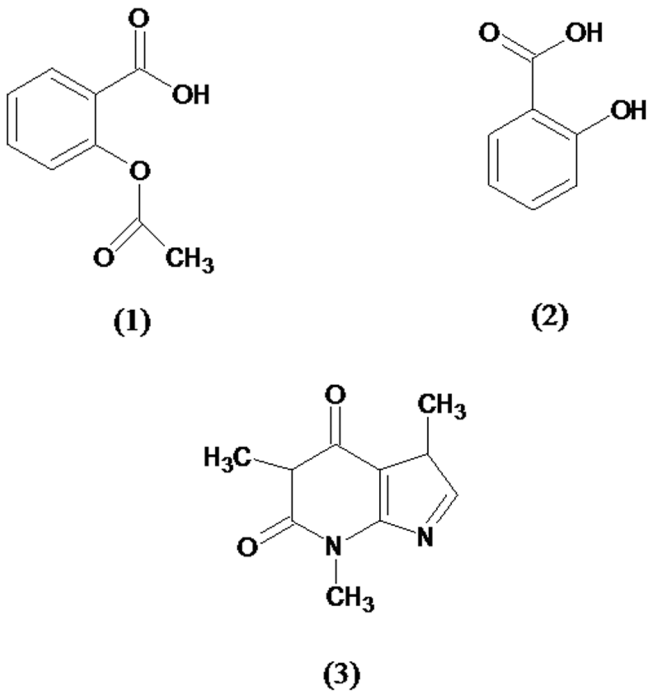

Fig. 1. Chemical structures of aspirin (1), salicylic acid (2), and caffeine (3).

The object of the present investigation is to develop a simple, rapid, economical, accurate, precise and stability-indicating spectrometric method for the analysis of ASA, SA, and CF as a single component and in mixtures. The developed method would be validated according to the guidelines of International Council for Harmonization (ICH)[95]. The proposed method would be used to study the degradation of ASA in the presence and absence of CF in the pH range of 8.0–10.0.

2. MATERIALS AND METHODS

2.1. Materials

ASA, SA, and CF were obtained from Sigma Aldrich (ST Louis, MD, USA). All the solvents used were of analytical grade purchased from Merck (Damsdat, Germany). The following buffer systems were used: 0.2 M HCl–KCl (pH 2.0) and 0.2 M H_3BO_3–NaOH (pH 9.0). Three different brands of ASA were purchased from the local market.

2.2. Methods

2.2.1. Measurement of pH

Elmetron LCD display pH meter (Model-CP501, sensitivity ±0.01 pH unit, Poland) was used for the determination of pH of the solutions using a combined electrode. The pH meter was calibrated using commercially available buffer tablets (Merck, Germany) of pH 4 and 7.

2.2.2. Selection of solvent and solution preparation

The solvent selection was based on the stability and solubility of drugs. For this purpose HCl–KCl 0.2 M buffer (pH 2.0) was used as a solvent for UV spectral determination. A standard stock solution of ASA, SA, and CF, each was prepared by dissolving 10 mg of each component in 100 ml of the buffer.

2.2.3. Thin-layer chromatography (TLC)

The TLC system used for the separation and identification of ASA and its degradation product, SA, included 250-µm cellulose plates (Merck, Germany) using the solvent systems:

a) methanol-strong ammonia solution (100:1.5, v/v), b) chloroform-acetone (40:100, v/v)[96]. The compounds were detected by their characteristic fluorescence on exposure to UV (254 and 365 nm) light.

2.2.4. Ultraviolet spectrometry

The determination of absorption spectra and measurement of absorbance of all the solutions was performed on a Thermoscientific UV–Vis spectrophotometer (Evolution 201, USA) using quartz cell of 10-mm path length. The absorbance scale was checked at suitable intervals using potassium dichromate as the calibration standard[97]. Solutions of ASA, SA, and CF were diluted in 0.2 M HCl–KCl buffer (pH 2.0) to prepare further dilutions. The absorbance of these solutions was measured in the range of 200–400 nm to locate the absorption maximum of ASA, SA, and CF.

2.2.5. Single and multicomponent spectrometric assay methods

2.2.5.1. Single-component assay

A 5 ml aliquot of ASA, SA, and CF as an individual compound was placed in 10 ml beaker and the pH of the solution was adjusted to 2.0 with few drops of 1.0 M HCl. This solution was transferred to a 10 ml volumetric flask and the volume was made up with 0.2 M KCl–HCl buffer. The absorbance of these solutions was determined at their respective absorption maxima and the concentrations of the ASA, SA, and CF were determined using the single-component spectrometric method in the linear calibration range.

2.2.5.2. Two-or three-component assay

A 5 ml aliquot of the mixtures (ASA and CF, ASA and SA, SA and CF, ASA, SA, and CF) was placed in 10 ml beaker and the pH of the solutions was adjusted to 2.0 with few drops of 1.0 M HCl. The volume of the solutions was made up with the KCl–HCl (0.2 M) buffer. The absorbance of the solutions was determined at the wavelengths of the absorption maxima of these compounds and the concentrations were determined using two or three-component spectrometric method.

2.2.5.3. Two-component assay of ASA/CF and ASA/SA mixtures

In a two-component spectrometric assay, absorbance measurements were performed at two appropriate wavelengths and the concentrations of the two components were determined by solving two simultaneous equations as follows:

$$A_1 = {}_1\epsilon_1\ {}_1C + {}_2\epsilon_1\ {}_2C \quad (1a)$$

$$A_2 = {}_2\epsilon_1\ {}_1C + {}_2\epsilon_2\ {}_2C \quad (1b)$$

where

${}_1\epsilon_1$ is absorptivity-cell path product for component 1 at wavelength λ_1

${}_1\epsilon_2$ is absorptivity-cell path product for component 1 at wavelength λ_2

${}_2\epsilon_1$ is absorptivity-cell path product for component 2 at wavelength λ_1

${}_2\epsilon_2$ is absorptivity-cell path product for component 2 at wavelength λ_2

${}_1C$ is the concentration of component 1

${}_2C$ is the concentration of component 2

The solutions of equation (1a) and (1b) for ${}_1C$ and ${}_2C$ are carried out as follows:

$${}_1C = ({}_2\epsilon_2\ A_1 - {}_2\epsilon_1\ A_2) / ({}_1\epsilon_1\ {}_2\epsilon_2 - {}_2\epsilon_1\ {}_1\epsilon_2) \quad (2a)$$

$${}_2C = ({}_1\epsilon_1\ A_2 - {}_1\epsilon_2\ A_1) / ({}_1\epsilon_1\ {}_2\epsilon_2 - {}_2\epsilon_1\ {}_1\epsilon_2) \quad (2b)$$

The same calculations have been applied to the use of specific absorbance [A (1%1cm)] as absorptivity in the above equations.

2.2.5.4. Three-component assay of ASA, SA, and CF mixtures

In a three-component assay, three simultaneous equations are solved by the use of a computer program. In this case for A_1, A_2, A_3 absorbance measurements at λ_1, λ_2, λ_3 for a mixture of components 1, 2, 3 having concentrations ${}_1C$, ${}_2C$, ${}_3C$, the following equations are developed:

Wavelength	Absorbance	Absorbance Sum
λ_1	$A_1\ {}_1\epsilon_1$	${}_1C + {}_2\epsilon_1\ {}_2C + {}_3\epsilon_1\ {}_3C$
λ_2	$A_2\ {}_1\epsilon_2$	${}_1C + {}_2\epsilon_2\ {}_2C + {}_3\epsilon_2\ {}_3C$
λ_3	$A_3\ {}_1\epsilon_3$	${}_1C + {}_2\epsilon_3\ {}_2C + {}_3\epsilon_3\ {}_3C$

(3a)

The matrix equation could be written as follows:

$$\begin{bmatrix} A_1 \\ A_2 \\ A_3 \end{bmatrix} = \begin{bmatrix} {}_1\epsilon^1 & {}_2\epsilon^1 & {}_3\epsilon^1 \\ {}_1\epsilon^2 & {}_2\epsilon^2 & {}_3\epsilon^2 \\ {}_1\epsilon^3 & {}_2\epsilon^3 & {}_3\epsilon^3 \end{bmatrix} \begin{bmatrix} {}_1C \\ {}_2C \\ {}_3C \end{bmatrix}$$

(AM) (ASM) (CM)

(3b)

where (AM) = Absorbance matrix
(ASM) = Absorbance sum matrix
(CM) = Concentration matrix

The solution of (3b) for each concentration ($_1C$, $_2C$, $_3C$) is carried out by replacing the appropriate column in the absorbance sum matrix in its determinant form and dividing the resultant by the absorbance sum matrix (ASM) again in its determinant form.

In a similar manner, the matrices are expanded for $_2C$ and $_3C$. For each determinant of a different set of $_1C$, $_2C$ and $_3C$ the top line of equation (5b) is computed fresh since A_1, A_2 and A_3 vary whilst ASM remains the same.

In the determination of mg% quantities of the compounds described in the above assay method, the term ϵ (molar absorptivity) is replaced by A (1% 1 cm), the specific absorbance of the compound in the equations.

2.2.6. Validation of the analytical method

USP[98] and ICH guideline[95] for analytical procedures

$$ {}_1C = \begin{vmatrix} A_1 {}_2\epsilon^1 & {}_3\epsilon^1 \\ A_2 {}_2\epsilon^2 & {}_3\epsilon^2 \\ A_3 {}_2\epsilon^3 & {}_3\epsilon^3 \end{vmatrix} \Big/ \begin{vmatrix} {}_1\epsilon^1 & {}_2\epsilon^1 & {}_3\epsilon^1 \\ {}_1\epsilon^2 & {}_2\epsilon^2 & {}_3\epsilon^2 \\ {}_1\epsilon^3 & {}_2\epsilon^3 & {}_3\epsilon^3 \end{vmatrix} \quad (4a)$$

$$ {}_2C = \begin{vmatrix} {}_1\epsilon^1 A_1 & {}_3\epsilon^1 \\ {}_1\epsilon^2 A_2 & {}_3\epsilon^2 \\ {}_1\epsilon^3 A_3 & {}_3\epsilon^3 \end{vmatrix} \Big/ \left|(ASM)\right| \quad (4b)$$

$$ {}_3C = \begin{vmatrix} {}_1\epsilon^1 {}_2\epsilon^1 A_1 \\ {}_1\epsilon^2 {}_2\epsilon^2 A_2 \\ {}_1\epsilon^3 {}_2\epsilon^3 A_3 \end{vmatrix} \Big/ \left|(ASM)\right| \quad (4c)$$

The matrices for $_1C$ are expanded by using the top row and Laplace's method

$$ {}_1C = \frac{A_1 \begin{vmatrix} {}_2\epsilon^2 & {}_3\epsilon^2 \\ {}_2\epsilon^3 & {}_3\epsilon^3 \end{vmatrix} - {}_2\epsilon^1 \begin{vmatrix} A_2 & {}_3\epsilon^2 \\ A_3 & {}_3\epsilon^3 \end{vmatrix} + {}_3\epsilon^1 \begin{vmatrix} A_2 & {}_2\epsilon^2 \\ A_3 & {}_2\epsilon^3 \end{vmatrix}}{\text{ASM expanded}} \quad (5a)$$

$$ {}_1C = \frac{A_1 ({}_2\epsilon^2 {}_3\epsilon^3 - {}_3\epsilon^2 {}_2\epsilon^3) - {}_2\epsilon^1 (A_2 {}_3\epsilon^3 - {}_3\epsilon^2 A_3) + {}_3\epsilon^1 (A_2 {}_2\epsilon^3 - {}_2\epsilon^2 A_3)}{\text{ASM expanded}} \quad (5b)$$

have provided a detailed description of validation parameters. These parameters are considered necessary before validation studies are conducted. The validation parameters studied are as follows:

2.2.6.1. Linearity and Range

The linearity of the developed method was evaluated by plotting the mean absorbencies of ASA, SA, and CF versus respective concentrations. The samples of ASA, CA and SA in the concentration range of 0.0005–0.005 mg% were prepared and the correlation coefficient, slope and intercept with standard deviation and error were calculated.

2.2.6.2. Accuracy

The accuracy of the method was evaluated by preparing six different concentrations (1–6 mg/100 ml) for ASA, SA, and CF and analyzing the solutions individually. The accuracy was also determined for the two-component and three-component mixtures of the samples. Each solution was prepared in triplicate and the % recovery was calculated.

2.2.6.3. Precision

The intra- and inter-day precisions were determined by preparing two different concentrations (0.002 and 0.005 mg/100 ml) of ASA, SA, and CF. The assay was performed at three different time intervals on the same day for intra-day while at three different days for inter-day precision and %RSDs were calculated.

2.2.6.4. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ (sensitivity) of the method was determined using following formulas:

$$LOD = 3.3 \times \frac{\sigma}{S}$$

$$LOQ = 10 \times \frac{\sigma}{S}$$

where σ is the standard deviation of the intercept of the regression line and S is the slope of the calibration curve.

2.2.6.5. Robustness

The robustness of the method was determined by making small changes in the wavelength (2 nm). The accuracy and precision of the method was evaluated.

2.2.7. Degradation of ASA

2.2.7.1. Degradation in solutions

A 10 mg per 100 ml aqueous solution of ASA was prepared (pH 9.0) in a 100 ml volumetric flask. The flask was placed in a water bath at 70°C to hydrolyze ASA. A 5 ml aliquot of the solution was pipette out at different intervals, cooled to room temperature in an ice bath and a portion was used for the assay of ASA and SA in the solution. The same experiment was repeated in the presence of CF and the contents containing ASA, SA, and CF were assayed in the solution, using the multicomponent spectrometric method (Section 2.2.5).

2.2.7.2. Degradation in tablets

Three different commercial brands of ASA tablets (75, 125, 300 mg) were placed in a desiccator containing a 22.5% NaOH solution to maintain an atmosphere of 65% relative humidity[99]. Samples of the three brands were withdrawn at appropriate intervals for the assay of ASA in degraded tablets.

3. RESULTS AND DISCUSSION

In the analysis of pharmaceutical mixtures and in drug degradation/stability studies, the analytical method used should be specific and stability-indicating to determine the intact drug and other components/products accurately[100]. Multicomponent spectrometric[4,14,101] and HPLC methods[61,102] are extensively used for this purpose. Since in many cases the drug concentration in a dosage form/medium is in low quantities, the method should be sensitive enough to determine the contents of the individual components in small quantities. The development of a stability-indicating method should be predicted by the intended application of the method and also a selection of appropriate technical design to assess the stability of active pharmaceutical ingredient. The application of a stability-indicating method involves monitoring of the stability of a drug in the final formulation which is required to evaluate the stability-indicating

characteristics of the method for the desired purpose. Some pharmaceutical companies to some extent still use a non-stability-indicating method such as UV spectrometry for product release and an HPLC method for stability testing. The chromatographic methods (TLC, GLC, and HPLC) are stability-indicating and stability-specific methods. However, the non-chromatographic, titrimetric and spectrometric techniques (UV, and IR) are not considered stability-indicating and are not suitable for application to the assessment of the stability of drugs. However, the multicomponent UV/visible spectrometric methods that take into consideration the presence of the intact drug as well as the degradation products may be applied to assess the stability of a drug during the degradation studies.

3.1. Spectral Characteristics

The UV absorption spectra of ASA, SA, and CF at pH 2.0 are shown in Fig. 2, which indicates their absorption maxima at 228, 303 and 272 nm, respectively. The value of A (1%, 1 cm) at the absorption maxima are given in Table 1 and are in agreement with the values reported by Moffat et al.[96].

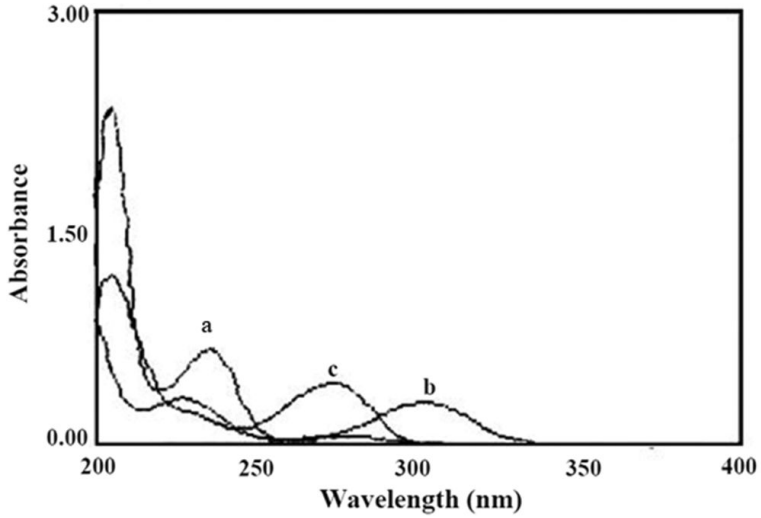

Fig. 2. Absorption spectra of ASA (a), SA (b) and CF (c) at pH 2.0 in 0.2 M KCl–HCl buffer.

Table 1. Specific absorbance [A (1% 1 cm)] ± SD values of ASA, SA and CF at pH 2.0 (KCl–HCl, 0.2 M) used in two- and three-component spectrometric assays[a]

Compound	228 nm	272 nm	278 nm	303 nm
ASA	461.16±1.08	58.76±0.15	67.40±0.40	9.16±0.20
SA	438.80± 0.76	78.20±0.64	98.66±0.57	259.16± 0.76
CF	278.50± 0.86	502.33±0.57	455.33±0.61	8.10±0.36

3.2. Validation of the Analytical Method

3.2.1. Linearity and range

The absorbance values of five different dilutions of ASA, SA, and CF (0.0005–0.005%), were measured and plotted against the respective concentrations. There was a linear relationship between the absorbance and concentration indicating the validity of Beer's Law in the concentration range studied (Fig. 3). The values of regression coefficient for ASA, SA, and CF are 0.9996, 0.9995 and 0.9997, respectively, and are given in Table 2.

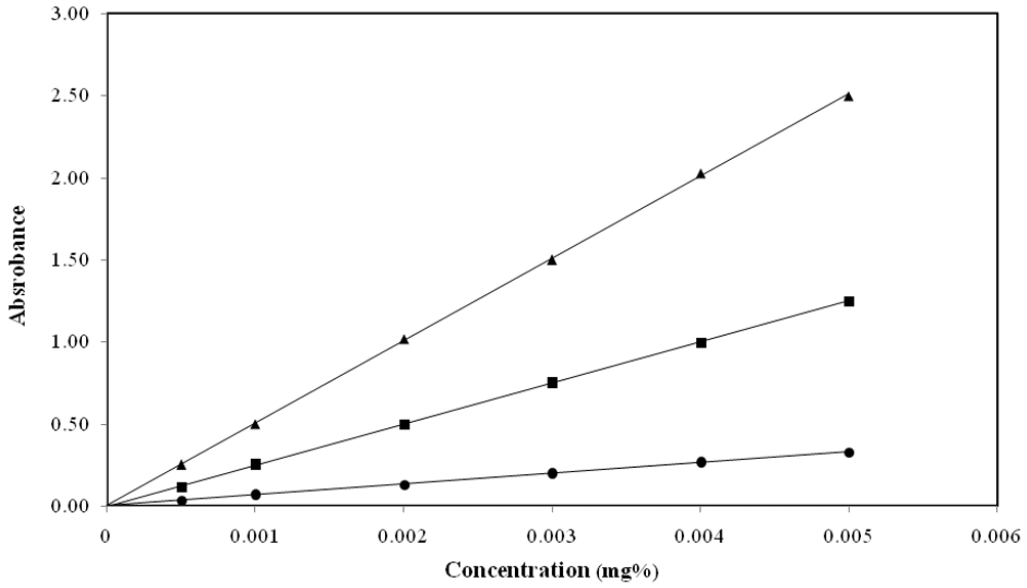

Fig. 3. Calibration curve of ASA (●), SA (■) and CF (▲) at a concentration range of 0.0005–0.005 mg%.

Table 2. Validation data for multicomponent spectrophotometric assay of aspirin (ASA), salicylic acid (SA) and caffeine (CF)[a]

Compound	ASA	SA	CF
λ_{max} nm (pH 2.0)	278	303	272
A (1%, 1 cm)	67.40±0.40	259.21±0.76	502.30±0.57
Linearity	0.9996	0.9995	0.9997
Concentration range (mg%)	0.0005–0.005	0.0005–0.005	0.0005–0.005
Slope	66.8	259.1	501.8
Intercept	0.0015	0.0003	0.0012
SE of Slope	0.0022	0.0027	0.0028
Recovery range (%)	98.3–103.2	98.0–105.0	98.0–100.2
Accuracy (%) ± SD	100.3±0.75	99.9±0.26	100.4±0.46
RSD (%)	0.98	2.30	1.41
LOD (mg%)	1.0×10^{-4}	3.4×10^{-5}	1.8×10^{-5}
LOQ (mg%)	3.2×10^{-4}	1.0×10^{-4}	5.5×10^{-5}

[a] Values are mean of five determinations.

3.2.2. Accuracy

The accuracy of the method has been determined by preparing solutions of ASA, SA, and CF individually and in mixtures and the results are given in Tables 3, 4 and 5, respectively. The data indicates the accuracy of the assay of compounds individually within ±2% deviation.

Table 3. The accuracy of the assay of ASA, SA and CF as individual compounds (n = 3)

Compound	Added (mg/100 ml)	Found (mg/100 ml)	Recovery (%)	RSD (%)
ASA	1.00	0.99	99.00	1.08
	2.00	1.98	99.00	0.98
	3.00	2.99	99.67	1.14
	4.00	4.00	100.00	1.21
	5.00	4.96	99.20	0.68
	6.00	5.99	99.83	0.59
SA	1.00	0.99	99.00	1.15
	2.00	1.97	98.50	0.75
	3.00	2.99	99.67	0.28
	4.00	3.99	99.75	1.04
	5.00	4.96	99.20	2.00
	6.00	5.94	99.00	0.98
CF	1.00	0.98	98.00	0.98
	2.00	2.00	100.00	0.58
	3.00	2.98	99.33	1.07
	4.00	3.99	99.75	0.96
	5.00	4.95	99.00	1.54
	6.00	5.98	99.67	1.12

Table 3. The accuracy of the assay of ASA, SA and CF in mixtures (Two-component) (n = 3)

Added (mg%)	Found (mg%)	Recovery (%)	RSD (%)	Added (mg%)	Found (mg%)	Recovery (%)	RSD (%)
ASA				**SA**			
8.00	7.97	99.62	0.12	0.40	0.40	100.00	0.57
6.00	5.89	98.16	0.22	0.80	0.80	100.00	0.46
5.00	5.01	100.20	0.75	1.00	1.00	100.00	0.26
4.00	3.96	99.00	0.64	1.20	1.19	99.16	0.24
2.00	1.97	98.50	0.29	1.60	1.59	99.40	0.46
1.00	1.00	100.00	0.57	1.80	1.81	100.56	0.65
SA				**CF**			
1.80	1.80	100.00	0.17	0.20	0.20	100.00	0.57
1.60	1.59	99.40	0.67	0.40	0.40	100.00	0.46
1.20	1.19	99.16	0.24	0.80	0.81	101.25	0.25
1.00	1.00	100.00	0.21	1.00	1.00	100.00	0.13
0.80	0.79	98.75	0.32	1.20	1.20	100.00	1.27
0.40	0.39	97.50	0.65	1.60	1.60	100.00	0.25
CF				**ASA**			
4.00	4.00	100.00	0.98	0.60	0.59	98.33	0.23
5.00	5.05	101.00	0.63	0.50	0.51	102.00	1.08
6.00	5.98	99.67	0.75	0.40	0.39	97.50	0.98
7.00	7.01	100.14	0.57	0.30	0.30	100.00	0.16
8.00	7.99	99.88	0.46	0.20	0.20	100.00	0.28
9.00	8.95	99.44	0.34	0.10	0.10	100.00	0.61

Table 5. The accuracy of the assay of ASA, SA, and CF in mixtures (three-component) (n = 3)

ASA				CF				SA			
Added (mg%)	**Found (mg%)**	**Recovery (%)**	**RSD (%)**	**Added (mg%)**	**Found (mg%)**	**Recovery (%)**	**RSD (%)**	**Added (mg%)**	**Found (mg%)**	**Recovery (%)**	**RSD (%)**
1.60	1.64	102.50	1.33	0.20	0.19	95.00	2.07	0.20	0.20	100.00	2.09
1.40	1.44	102.96	1.39	0.40	0.40	100.00	1.41	0.20	0.20	100.00	0.29
1.20	1.19	99.17	0.51	0.60	0.59	98.33	1.16	0.20	0.19	95.00	0.76
1.00	0.98	98.00	1.57	0.60	0.59	98.33	0.76	0.40	0.38	95.00	2.21
0.60	0.60	100.00	1.18	0.80	0.79	98.75	0.75	0.60	0.60	100.00	0.91
0.40	0.39	97.50	1.14	0.40	0.39	97.50	0.30	1.20	1.19	99.17	0.45
0.20	0.19	95.00	2.15	1.40	1.39	99.29	1.05	0.40	0.40	100.00	1.41
0.60	0.59	98.33	1.30	1.20	1.19	99.17	0.84	0.20	0.20	100.00	2.30
0.80	0.79	98.75	1.58	0.60	0.59	98.33	1.15	0.60	0.60	100.00	1.95
1.00	0.98	98.00	1.16	0.20	0.20	100.00	1.15	0.80	0.80	100.00	1.26

Table 6. Intra- and inter-day precisions of the assay of ASA, SA, and CF as individual compounds

Sample	**Concentration (mg%)**	**Amount measured (mg%)**			**Mean (mg%)**	**Recovery (%)**	**RSD (%)**
Intra-day Precision		**10 am**	**1 pm**	**4 pm**			
ASA	0.0050	0.0050	0.0049	0.0048	0.0049	98.00	2.04
	0.0021	0.0021	0.0020	0.0020	0.0020	100.00	2.84
SA	0.0051	0.0051	0.0050	0.0048	0.0049	98.00	3.08
	0.0021	0.0021	0.0020	0.0021	0.0020	100.00	2.79
CF	0.0051	0.0051	0.0051	0.0049	0.0050	100.00	2.29
	0.0020	0.0020	0.0019	0.0020	0.0019	95.00	2.99
Intermediate Precision		**1st day**	**2nd day**	**3rd day**			
ASA	0.0050	0.0050	0.0048	0.0045	0.0048	96.00	2.00
	0.0020	0.0020	0.0019	0.0020	0.0020	100.00	2.94
SA	0.0050	0.0050	0.0049	0.0051	0.0050	100.00	2.00
	0.0020	0.0020	0.0019	0.0020	0.0020	100.00	2.94
CF	0.0050	0.0050	0.0049	0.0051	0.0050	100.00	2.00
	0.0020	0.0020	0.0018	0.0020	0.0019	95.00	2.79

3.2.3. Precision

The precision (repeatability) of the assay method for ASA, SA and CF has been determined as follows:

3.2.3.1. Intra-day precision (Repeatability)

Six independent assays of ASA, SA, and CF were performed at two different concentrations (0.002 and 0.005%) and absorbance measurements were made at 3 h intervals. The results are reported in Table 6.

3.2.3.2. Inter-day precision (Intermediate precision)

It has also been carried out on the above mentioned two solutions for 3 days and the results are reported in Table 6.

3.2.5. Limit of detection (LOD) and limit of quantification (LOQ)

LOD is the lowest amount of an analyte that can be detected. This was determined by measuring the absorbance of six different solutions in the concentration range of 0.0005–0.005 mg% for each compound (ASA, SA, CF). It has been found that the LODs of the method for ASA, SA, and CF are 1.0×10^{-4}, 3.4×10^{-5} and 1.8×10^{-5} mg%, respectively. Similarly, the lowest amount of the analyte that can be quantitatively determined with suitable precision is termed as LOQ. It has been found that the LOQs of the method for ASA, SA, and CF are 3.2×10^{-4}, 1.0×10^{-4} and 5.5×10^{-5} mg%, respectively. Both LOD and LOQ values are given in Table 2.

3.2.7. Robustness

Robustness of a method is described as a measure of the capability of an assay method to remain unchanged by small variations in the parameters. The absorbance measurements of the solutions of ASA, SA, and CF carried out at the respective wavelengths did not show any significant variations in the results (0.1–0.5%). Therefore, the method is found to be robust in its use for the assay of the desired compounds.

3.3. Assay of ASA and SA in Degraded Solutions

According to USP[98], the specificity of an assay method is its ability to measure an analyte accurately and specifically in the presence of other components in a sample mixture such as other active ingredients, excipients, related compounds, impurities and degradation products. The present methods are based on the assay of two- or three-component mixtures including ASA and degradation product, SA, in the presence and absence of CF in thermally degraded solutions of ASA and in the commercial tablets of ASA exposed to 65% relative humidity.

The assay data of ASA at various time intervals in the absence and presence of CF are reported in Tables 7 and 8, respectively. In order to observe the specificity of the assay method, the data were plotted as log concentration versus time and found to follow first-order kinetics in the absence (Fig. 4a) and presence of CF (Fig. 4b). The data appeared to fit well in the first order-plot indicating a gradual loss of ASA with time. The values of apparent first-order rate constants (k_{obs}) for these reactions are given in Table 9. Thus the linearity of the curve around the assay data is a measure of the specificity of the method ($R^2 = 0.9996$). This shows that SA is also accounted in the assay method (two-component as ASA and SA) and the method could be considered specific for the degradation studies of ASA.

A typical kinetic plot for the degradation of ASA and the formation of SA is given in Fig. 5. The values of k_{obs} increase with pH indicating an increase in the rate of hydrolysis of ASA in the absence of CF as compared to that of in presence of CF (Fig. 6). The decrease in the rate of hydrolysis in the presence of CF is most probably due to some interaction of ASA with CF which is less susceptible to the hydrolysis[103]. The degradation of ASA has also been carried out at pH 8 and 10 and similar results have been obtained. Each solution also contained CF but the results for three-component assay indicated non-interference of SA and CF in the method.

Table 7. Degradation of ASA in the absence of CF at pH 8.0 to 10.0

Time (min)	ASA (mg%)	SA (mg%)
pH 8.0		
0	5.00	0.00
10	4.77	0.29
20	4.54	0.50
30	4.31	0.72
40	4.08	0.93
50	3.85	1.14
60	3.62	1.35
pH 9.0		
0	5.00	0.00
10	4.78	0.27
20	4.46	0.53
30	4.16	0.90
40	3.93	1.11
50	3.60	1.48
60	3.27	1.75
pH 10.0		
0	5.00	0.00
10	4.66	0.36
20	4.24	0.86
30	3.89	1.22
40	3.42	1.65
50	3.00	2.21
60	2.59	2.59

Table 8. Degradation of ASA in the presence of CF at pH 8.0 to 10.0

Time (min)	ASA (mg%)	CF (mg%)	SA (mg%)
pH 8.0			
0	5.00	0.80	0.00
10	4.78	0.80	0.22
20	4.57	0.80	0.40
30	4.31	0.80	0.65
40	4.16	0.80	0.87
50	3.89	0.80	1.09
60	3.71	0.80	1.31
pH 9.0			
0	5.00	0.80	0.00
10	4.75	0.80	0.20
20	4.50	0.80	0.40
30	4.16	0.80	0.69
40	3.71	0.80	1.26
50	3.54	0.80	1.54
60	3.31	0.80	1.75
pH 10.0			
0	5.00	0.80	0.00
10	4.46	0.80	0.49
20	3.98	0.80	0.98
30	3.54	0.80	1.45
40	3.31	0.80	1.78
50	2.95	0.80	1.99
60	2.63	0.80	2.32

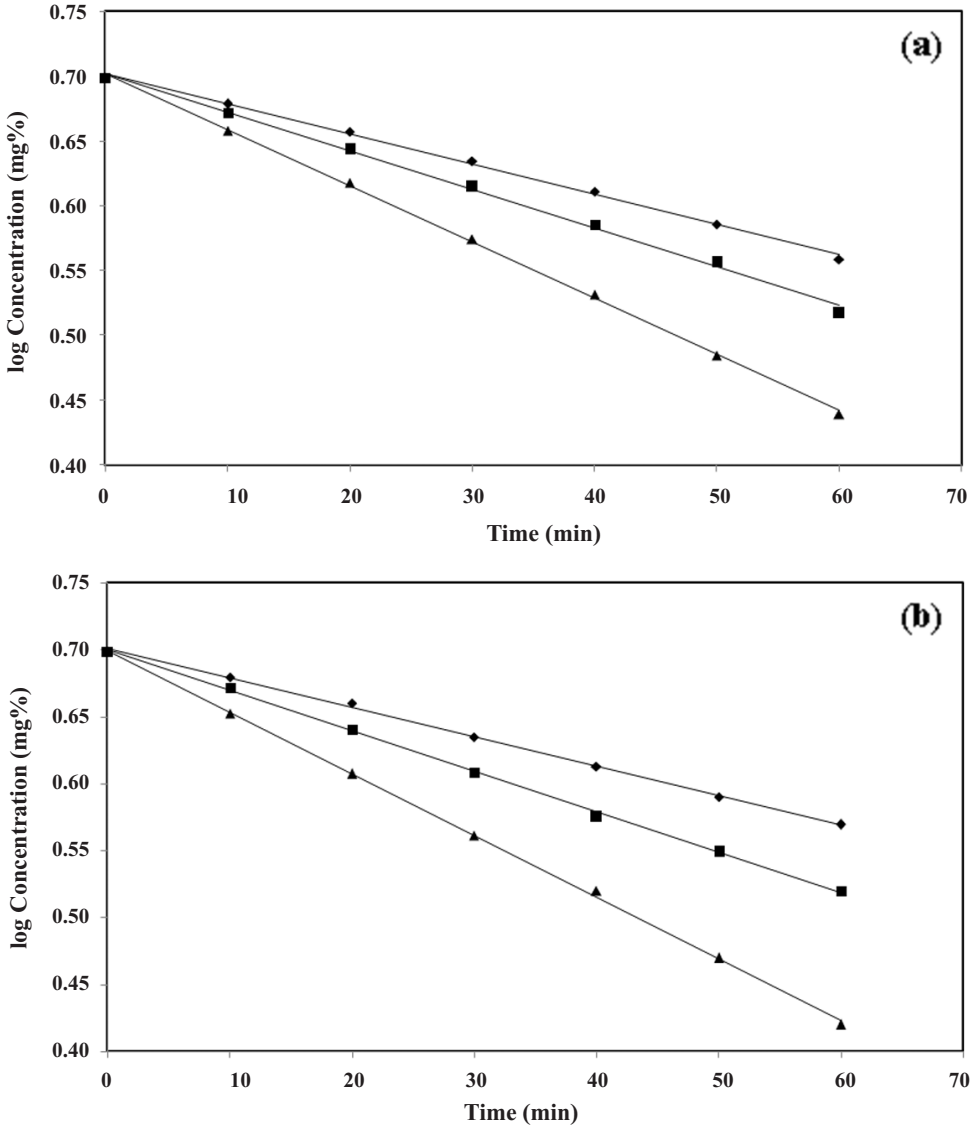

Fig. 4. First-order plots for the hydrolysis of ASA in the absence (a) and presence (b) of CF at pH 8.0 (●), 9.0 (■) and 10.0 (▲).

Table 9. Apparent first-order rate constants (k_{obs}) for the degradation of aspirin (70^0C) in the absence and presence of CF

pH	$k_{obs} \times 10^{-3} min^{-1}$	
	Absence of CF	Presence of CF
8.0	5.37	4.99
9.0	6.90	6.52
10.0	11.00	10.30

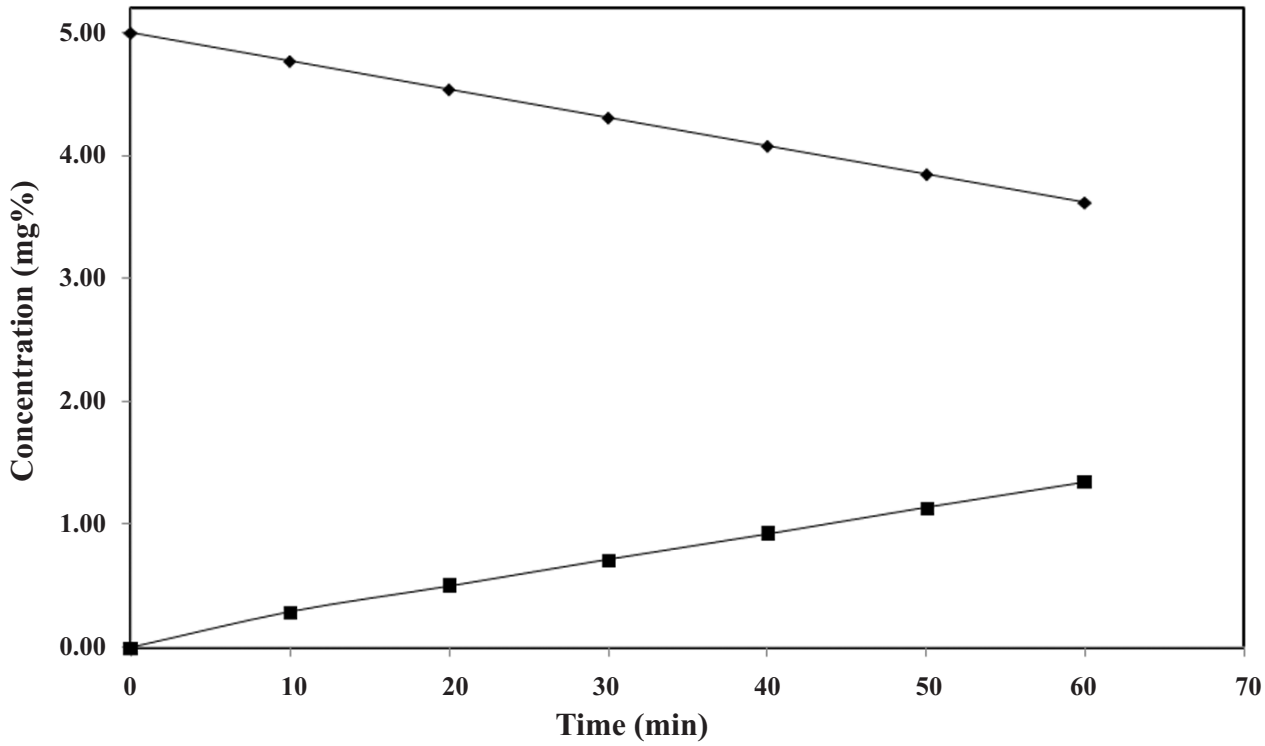

Fig. 5. Kinetic plots for the hydrolysis of ASA (♦) and formation of SA (■) at pH 9.0.

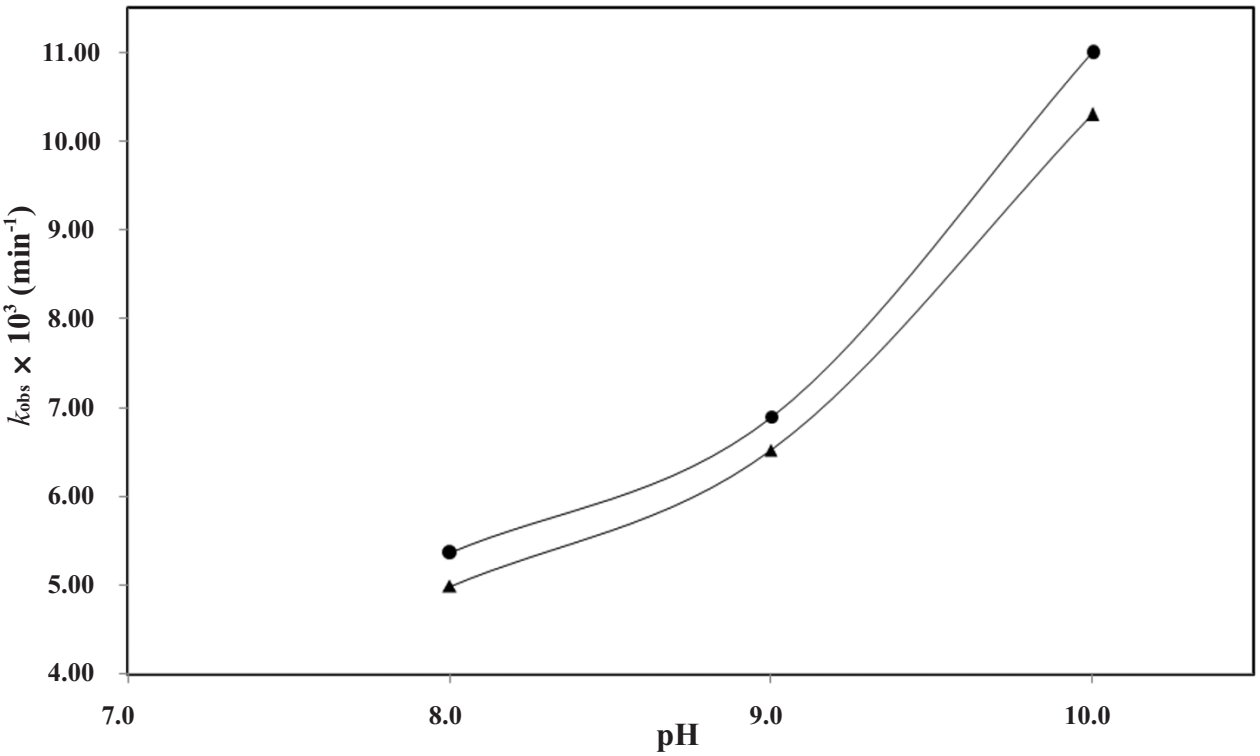

Fig. 6. *k*-pH profiles for the hydrolysis of ASA in the absence (●) and presence (▲) of CF at pH 8.0–10.0.

3.4. Assay of ASA in Degraded Tablets

A total of three different brands of ASA tablets stored in an atmosphere of 65% relative humidity were assayed at different time intervals (Table 10) and the data was plotted as log concentration versus time (figure not shown). Similar to the degradation of ASA in aqueous solutions (section 2.2.7), the assay data was found to comply with the first-order kinetics (similar to Fig. 4), showing the linearity of the plot around the assay values. This indicates that the assay method is reliable giving accurate results with decreasing concentration of ASA as observed from the data fitting of the plots. The values of the apparent first-order rate constants (k_{obs}) for the degradation of ASA in commercial tablets are given in Table 11. Thus the assay method is stability-indicating and can be conveniently applied to the stability analysis of ASA.

Table 10. Degradation of commercial aspirin tablets in different brands

Time (days)	Brand 1	Brand 2	Brand 3
	Concentration (mg/Tab)	Concentration (mg/Tab)	Concentration (mg/Tab)
0	96.6	98.1	100.3
2	78.6	94.0	96.6
4	69.6	89.9	92.9
6	60.6	85.8	89.2
8	51.5	81.7	85.5
10	42.4	77.6	81.8

Table 11. Apparent first-order rate constants for the degradation of aspirin in commercial tablets

Brand	$k_{obs} \times 10^2$, day^{-1}	t_{90} (days)
1	8.01	1.3
2	2.52	4.2
3	2.05	5.1

4. CONCLUSION

A simple, rapid and stability-indicating multicomponent (two- and three-component) spectrophotometric assay method is developed and validated for the assay of ASA and its degradation product, SA, in the presence and absence of CF. Commercially available ASA and ASA-CF preparations are in common use and could be easily analyzed by the proposed methods. In these methods, the simultaneous assay of these drugs in mixtures is carried out by absorbance measurements at two or three appropriately selected wavelengths and determination of the components by solving matrix equations. The methods have taken into consideration all the parameters necessary for their validation. In order to examine the specificity of these methods, they have been applied to ASA degradation studies and satisfactory results have been obtained when the assay data are subjected to kinetic treatment. These methods could thus be considered as specific

and stability-indicating for drug degradation studies of ASA. In the present methods, no interference is caused by the degradation product (SA) and added CF since these are also simultaneously assayed by these methods. These methods could also be conveniently used in a quality control laboratory and for stability analysis of ASA preparations.

FUNDING
None mentioned.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ETHICAL APPROVAL
Not applicable.

REFERENCES

1. Feher G, Feher A, Pusch G, Koltai K, Tibold A, Gasztonyi B, Papp E, Szapary L, Kesmarky G, Toth K. Clinical importance of aspirin and clopidogrel resistance. World J Cardiol. 2010;2:171-186.
2. Aydinalp A, Atar I, Gulmez O, Atar A, Acikel S, Bozbas H, Ozgul A, Ertan C, Ozin B, Muderrisoglu H. The clinical significance of aspirin resistance in patients with chest pain. Clin Cardiol. 2010;33:E1–E7.
3. Dai Y, Ge J. Clinical use of aspirin in treatment and prevention of cardiovascular disease. Thrombosis. 2011;2012:245037.
4. Glombitza BW, Schmidt PC. Comparison of three new spectrophotometric methods for simultaneous determination of aspirin and salicylic acid in tablets without separation of pharmaceutical excipients. J Pharm Sci. 1994;83:751-757.
5. Blondino FE, Byron PR. The quantitative determination of aspirin and its degradation products in a model solution aerosol. J Pharm Biomed Anal. 1995;13:111-119.
6. Kokot Z, Burda K. Simultaneous determination of salicylic acid and acetylsalicylic acid in aspirin delayed-release tablet formulations by second-derivative UV spectrophotometry. J Pharm Biomed Anal. 1998;18:871-875.
7. Schmidt PC, Glombitza BW. Quantitative multicomponent analysis of aspirin and salicylic acid in tablets without separation of excipients by means of principal component regression and a classical least squares algorithm. TrAC Trends Anal Chem. 1995;14:45-49.
8. Ahmed M, Biswas MH, Rahman MM, Bhuiyan MS, Kamal MA, Sadik G. Development of a spectrophotometric method for the determination of aspirin in blood sample. J Med Sci. 2001;1:61-62.
9. Ruiz-Medina A, Fernandez-de Cordova ML, Ortega-Barrales P, Molina-Diaz A. Flow-through UV spectrophotometric sensor for determination of (acetyl) salicylic acid in pharmaceutical preparations. Int J Pharm. 2001;216:95-104.
10. Burge LJ, Raches DW. A Rapid HPLC Assay for the Determination of dextropropoxyphene related substances in combination with aspirin, acetaminophen, and caffeine in tablet and capsule formulations. J Liq Chromatogr Relat Technol. 2003;26:1977-1990.
11. Matias FA, Vila MM, Tubino M. Quantitative reflectance spot test for the determination of acetylsalicylic acid in pharmaceutical preparations. J Braz Chem Soc. 2004;15: 327-330.
12. Dou Y, Mi H, Zhao L, Ren Y, Ren Y. Radial basis function neural networks in non-destructive determination of compound aspirin tablets on NIR spectroscopy. Spectrochim Acta A: Mol Biomol Spect. 2006;65:79-83.
13. Mishra P, Dolly A. Simultaneous determination of clopidogrel and aspirin in pharmaceutical dosage forms. Indian J Pharm Sci. 2006;68: 365-368.
14. Mot AC, Soponar F, Medvedovici A, Sârbu C. Simultaneous spectrophotometric determination of aspirin, paracetamol, caffeine, and chlorphenamine from pharmaceutical formulations using multivariate regression methods. Anal Lett. 2010;43:804-813.
15. Walter LJ, Biggs DF, Coutts RT. Simultaneous GLC estimation of salicylic acid and aspirin in plasma. J Pharm Sci. 1974;63:1754-1758.

16. Pedersen AK, Fitzgerald GA. Preparation and analysis of deuterium-labeled aspirin: Application to pharmacokinetic studies. J Pharm Sci. 1985;74:188-192.
17. Tsikas D, Tewes KS, Gutzki FM, Schwedhelm E, Greipel J, Frolich JC. Gas chromatographic-tandem mass spectrometric determination of acetylsalicylic acid in human plasma after oral administration of low–dose aspirin and guaimesal. J Chromatogr B: Biomed Sci Applic. 1998;709:79-88.
18. Lange WE, Bell SA. Fluorometric determination of acetylsalicylic acid and salicylic acid in blood. J Pharm Sci. 1966;55:386-389.
19. Street KW, Schenk GH. Spectrofluorometric determination of acetylsalicylic acid, salicylamide, and salicylic acid as an impurity in pharmaceutical preparations. J Pharm Sci. 1981;70:641-646.
20. Konstantianos DG, Ioannou PC, Efstathiou CE. Simultaneous determination of acetylsalicylic and salicylic acids in human serum and aspirin formulations by second-derivative synchronous fluorescence spectrometry. Analyst. 1991;116:373-378.
21. Villari A, Micali N, Fresta M, Puglisi G. Simultaneous spectrophotometric determination in solid phase of aspirin and its impurity salicylic acid in pharmaceutical formulations. J Pharm Sci. 1992;81:895-898.
22. Konstantianos DG, Ioannou PC. Simultaneous determination of diflunisal and salicylic acid in human serum by second–derivative synchronous fluorescence spectroscopy. Eur J Pharm Sci. 1994;1:209-217.
23. Navalón A, Blanc R, del Olmo M, Vilchez JL. Simultaneous determination of naproxen, salicylic acid and acetylsalicylic acid by spectrofluorimetry using partial least–squares (PLS) multivariate calibration. Talanta. 1999;48:469-475.
24. Martos NR, Diaz AM, Navalon A, Capitan-Vallvey LF. Spectrofluorimetric determination of acetylsalicylic acid and codeine mixtures in pharmaceuticals. Anal lett. 2001;34:579-595.
25. Taguchi VY, Cotton ML, Yates CH, Millar JF. Determination of drug stability in aspirin tablet formulations by high-pressure liquid chromatography. J Pharm Sci. 1981;70:64-67.
26. Shen J, Wanwimolruk S, Roberts MS, Clark CR. A sensitive assay for aspirin and its metabolites using reversed-phase ion-pair high-performance liquid chromatography. J Liq Chromatogr. 1990;13:751-761.
27. Venema DP, Hollman PC, Janssen KP, Katan MB. Determination of acetylsalicylic acid and salicylic acid in foods, using HPLC with fluorescence detection. Journal Agric Food Chem. 1996;44:1762-1767.
28. Lo LY, Bye A. Specific and sensitive method for the determination of aspirin and salicylic acid in plasma using reversed-phase high-performance liquid chromatography. J Chromatogr B: Biomed Sci Applic. 1980;181:473-477.
29. McMahon GP, Kelly MT. Determination of aspirin and salicylic acid in human plasma by column-switching liquid chromatography using on-line solid-phase extraction. Anal Chem. 1998;70:409-414.
30. Gandhimathi M, Ravi TK, Abraham A, Thomas R. Simultaneous determination of aspirin and isosorbide 5-mononitrate in formulation by reversed phase high pressure liquid chromatography. J Pharm Biomed Anal. 2003;32:1145-1148.
31. Yamamoto E, Takakuwa S, Kato T, Asakawa N. Sensitive determination of aspirin and its metabolites in plasma by LC-UV using on-line solid-phase extraction with methylcellulose-immobilized anion-exchange restricted access media. J Chromatogr B. 2007;846:132-138.
32. An K, Ayyappan T, Raman VR, Vetrichelvan T, Sankar AS, Nagavalli D. RP-HPLC analysis of aspirin and clopidogrel bisulphate in combination. Indian J Pharm Sci. 2007;69:597-599.
33. Shah DA, Bhatt KK, Mehta RS, Shankar MB, Baldania SL. Development and validation of a RP-HPLC method for determination of atorvastatin calcium and aspirin in a capsule dosage form. Indian J Pharm Sci. 2007;69:546-549.

34. Wang N, Xu F, Zhang Z, Yang C, Sun X, Li J. Simultaneous determination of dipyridamole and salicylic acid in human plasma by high performance liquid chromatography-mass spectrometry. Biomed Chromatogr. 2008;22: 149-156.
35. Akay C, Degim IT, Sayal A, Aydin A, Ozkan Y, Gul H. Rapid and simultaneous determination of acetylsalicylic acid, paracetamol, and their degradation and toxic impurity products by HPLC in pharmaceutical dosage forms. Turkish J Med Sci. 2008;38:167-173.
36. Hammud HH, El Yazbib FA, Mahrous ME, Sonji GM, Sonji NM. Stability-indicating spectrofluorimetric and RP-HPLC methods for the determination of aspirin and dipyridamole in their combination. Open Spectrosc J. 2008;2:19-28.
37. Vora DN, Kadav AA. Validated ultra HPLC method for the simultaneous determination of atorvastatin, aspirin, and their degradation products in capsules. J Liq Chromatogr Relat Technol. 2008;31:2821-2837.
38. Xu X, Koetzner L, Boulet J, Maselli H, Beyenhof J, Grover G. Rapid and sensitive determination of acetylsalicylic acid and salicylic acid in plasma using liquid chromatography-tandem mass spectrometry: application to pharmacokinetic study. Biomed Chromatogr. 2009;23:973-979.
39. Chaudhary A, Wang J, Prabhu S. Development and validation of a high-performance liquid chromatography method for the simultaneous determination of aspirin and folic acid from nano-particulate systems. Biomed Chromatogr. 2010;24:919-925.
40. Coolen SA, Huf FA, Reijenga JC. Determination of free radical reaction products and metabolites of salicylic acid using capillary electrophoresis and micellar electrokinetic chromatography. J Chromatogr B: Biomed Sci Applic. 1998;717:119-124.
41. Hansen SH, Jensen ME, Bjornsdottir I. Assay of acetylsalicylic acid and three of its metabolites in human plasma and urine using non–aqueous capillary electrophoresis with reversed electroosmotic flow. J Pharm Biomed Anal. 1998;17:1155-1160.
42. Haque A, Stewart JT. Simultaneous determination of codeine, caffeine, butalbital, and aspirin by free solution capillary electrophoresis. J Liq Chromatogr Relat Technol. 1999;22:1193-1204.
43. Quintino MD, Corbo D, Bertotti M, Angnes L. Amperometric determination of acetylsalicylic acid in drugs by batch injection analysis at a copper electrode in alkaline solutions. Talanta. 2002;58:943-949.
44. Chen HL, Fan LY, Chen XG, Hu ZD, Zhao ZF, Hooper M. On-line conversion and determination of aspirin using a flow injection–capillary electrophoresis system. J Sep Sci. 2003;26: 863-868.
45. Fogg AG, Ali A, Abdalla MA. On-line bromimetric determination of phenol, aniline, aspirin and isoniazid using flow injection voltammetry. Analyst. 1983;108:840-846.
46. Wei W, Nie L, Yao S. Multi-component analysis in solution using piezoelectric quartz sensors: Part II. Determination of aspirin and salicylic acid in aqueous solutions. Anal Chim Acta. 1992;263:77-83.
47. Issopoulos PB. Micelle-assisted dissolution for the analysis of aspirin by second-order derivative potentiometry. Fresenius J Anal Chem. 1997;358:663-666.
48. Sartori ER, Medeiros RA, Rocha-Filho RC, Fatibello-Filho O. Square-wave voltammetric determination of acetylsalicylic acid in pharmaceutical formulations using a boron-doped diamond electrode without the need of previous alkaline hydrolysis step. J Brazilian Chem Soc. 2009;20:360-366.
49. Sanghavi BJ, Srivastava AK. Simultaneous voltammetric determination of acetaminophen, aspirin and caffeine using an in situ surfactant-modified multiwalled carbon nanotube paste electrode. Electrochim Acta. 2010;55:8638-8648.
50. Smith J. Anti-viral compositions and methods of making and using the anti-viral compositions. United States Patent US 20050003020A1, 2004.

51. Plaisance KI, Mackowiak PA. Antipyretic therapy: physiologic rationale, diagnostic implications, and clinical consequences. Arch Intern Med. 2000;160:449-456.
52. Gharib FA. Effect of salicylic acid on the growth, metabolic activities and oil content of basil and marjoram. Int J Agric Biol. 2006;4:485-492.
53. Steele K, Shirodaria P, O'Hare M, Merrett JD, Irwin WG, Simpson DI, Pfister H. Monochloroacetic acid and 60% salicylic acid as a treatment for simple plantar warts: effectiveness and mode of action. British J Dermatol. 1988;118:537-544.
54. Tinker RB, McBay AJ. Spectrophotometric determination of acetylsalicylic and salicylic acids. J Pharm Sci. 1954;43:315-317.
55. Kitamura K, Majima R. Determination of salicylic acid in aspirin powder by second derivative ultraviolet spectrometry. Anal Chem. 1983;55:54-56.
56. Saha U, Baksi K. Spectrophotometric determination of salicylic acid in pharmaceutical formulations using copper (II) acetate as a colour developer. Analyst. 1985;110:739-741.
57. Salinas F, de La Pena AM, Durán–Merás I, Durán MS. Determination of salicylic acid and its metabolites in urine by derivative synchronous spectrofluorimetry. Analyst. 1990;115:1007-1011.
58. Peng GW, Gadalla MA, Smith V, Peng A, Chiou WL. Simple and rapid high-pressure liquid chromatographic simultaneous determination of aspirin, salicylic acid, and salicyluric acid in plasma. J Pharm Sci. 1978;67:710-712.
59. Amick EN, Mason WD. Determination of aspirin, salicylic acid, salicyluric acid, and gentisic acid in human plasma and urine by high pressure liquid chromatography. Anal Lett. 1979;12:629-640.
60. Wahlin-Boll E, Brantmark B, Hanson A, Melander A, Nilsson C. High-pressure liquid chromatographic determination of acetylsalicylic acid, salicylic acid, diflunisal, indomethacin, indoprofen and indobufen. Eur J Clin Pharmacol. 1981;20:375-378.
61. Kees F, Jehnich D, Grobecker H. Simultaneous determination of acetylsalicylic acid and salicylic acid in human plasma by high–performance liquid chromatography. J Chromatogr B: Biomed Sci Applic. 1996;677:172-177.
62. Jian-Hua L, Smith PC. Direct analysis of salicylic acid, salicyl acyl glucuronide, salicyluric acid and gentisic acid in human plasma and urine by high-performance liquid chromatography. J Chromatogr B: Biomed Sci Applic. 1996;675:61-70.
63. Venema DP, Hollman PC, Janssen KP, Katan MB. Determination of acetylsalicylic acid and salicylic acid in foods, using HPLC with fluorescence detection. J Agric Food Chem. 1996;44:1762-1767.
64. Pirola R, Bareggi SR, De Benedittis G. Determination of acetylsalicylic acid and salicylic acid in skin and plasma by high-performance liquid chromatography. J Chromatogr B: Biomed Sci Applic. 1998;705:309-315.
65. Aboul-Soud MA, Cook K, Loake GJ. Measurement of salicylic acid by a high-performance liquid chromatography procedure based on ion-exchange. Chromatographia. 2004;59:129-133.
66. Rowland M, Riegelman S. Determination of acetylsalicylic acid and salicylic acid in plasma. J Pharm Sci. 1967;56:717-720.
67. Rance MJ, Jordan BJ, Nichols JD. A simultaneous determination of acetylsalicylic acid, salicylic acid and salicylamide in plasma by gas liquid chromatography. J Pharm Pharmacol. 1975;27:425-429.
68. Torriero AA, Luco JM, Sereno L, Raba J. Voltammetric determination of salicylic acid in pharmaceuticals formulations of acetylsalicylic acid. Talanta. 2004;62:247-254.
69. Nehlig A, Daval JL, Debry G. Caffeine and the central nervous system: mechanisms of action, biochemical, metabolic and psychostimulant effects. Brain Res Rev. 1992;17:139-170.
70. Bolton S, Null G. Caffeine: Psychological effects, use and abuse. Orthomol Psych.

1981;10:202-211.
71. Van Dongen H, Kerkhof G. Effects of caffeine on sleep and cognition. Prog Brain Res. 2011;190:105-117.
72. Bishop D. Dietary supplements and team-sport performance. Sports Med. 2010;40:995-1017.
73. Tarnopolsky MA. Caffeine and creatine use in sport. Ann Nutr Metab. 2010;57:1-8.
74. Schmidt B, Roberts RS, Davis P, Doyle LW, Barrington KJ, Ohlsson A, Solimano A, Tin W. Long-term effects of caffeine therapy for apnea of prematurity. N Engl J Med. 2007;357: 1893-1902.
75. Bozdogan A, Acar AM, Kunt GK. Simultaneous determination of acetaminophen and caffeine in tablet preparations by partial least–squares multivariate spectrophotometric calibration. Talanta. 1992;39:977-979.
76. Muszalska I, Zajac MA, Wrobel GR, Nogowska M. UV/VIS spectrophotometric methods for determination of caffeine and phenylephrine hydrochloride in complex pharmaceutical preparations. Validation of the methods. Acta Pol Pharm. 2000;57:247-252.
77. Satinsky D, Neto I, Solich P, Sklenarova H, Conceicao M, Montenegro BS, Araujo AN. Sequential injection chromatographic determination of paracetamol, caffeine, and acetylsalicylic acid in pharmaceutical tablets. J Sep Sci. 2004;27:529-536
78. Abbaspour A, Mirzajani R. Simultaneous determination of phenytoin, barbital and caffeine in pharmaceuticals by absorption (zero-order) UV spectra and first-order derivative spectra-multivariate calibration methods. J Pharm Biomed Anal. 2005;38:420-427.
79. Belay A, Ture K, Redi M, Asfaw A. Measurement of caffeine in coffee beans with UV/vis spectrometer. Food Chem. 2008;108:310-315.
80. Khoshayand MR, Abdollahi H, Shariatpanahi M, Saadatfard A, Mohammadi A. Simultaneous spectrophotometric determination of paracetamol, ibuprofen and caffeine in pharmaceuticals by chemometric methods. Spectrochim Acta Part A: Mol Biomol Spectrosc. 2008;70:491-499.
81. Belay A, Gholap AV. Characterization and determination of chlorogenic acids (CGA) in coffee beans by UV-Vis spectroscopy. AJPAC. 2009;3:234-240.
82. Atomssa T, Gholap AV. Characterization of caffeine and determination of caffeine in tea leaves using UV-visible spectrometer. Afr J Pure Appl Chem. 2011;5:1-8.
83. Aranda M, Morlock G. Simultaneous determination of riboflavin, pyridoxine, nicotinamide, caffeine and taurine in energy drinks by planar chromatography–multiple detection with confirmation by electrospray ionization mass spectrometry. J Chromatogr A. 2006;1131:253-260.
84. Castro J, Pregibon T, Chumanov K, Marcus RK. Determination of catechins and caffeine in proposed green tea standard reference materials by liquid chromatography-particle beam/electron ionization mass spectrometry (LC-PB/EIMS). Talanta. 2010;82:1687-1695.
85. Daghbouche Y, Garrigues S, Vidal MT, de la Guardia M. Flow injection Fourier transform infrared determination of caffeine in soft drinks. Anal Chem. 1997;69:1086-1091.
86. Singh BR, Wechter MA, Hu Y, Lafontaine C. Determination of caffeine content in coffee using Fourier transform infra-red spectroscopy in combination with attenuated total reflectance technique: a bioanalytical chemistry experiment for biochemists. Biochemical Educ. 1998;26: 243-247.
87. Bouhsain Z, Garrigues JM, Garrigues S, de la Guardia M. Flow injection Fourier transform infrared determination of caffeine in coffee. Vib Spectrosc. 1999;21:143-150.
88. Paradkar MM, Irudayaraj J. Rapid determination of caffeine content in soft drinks using FTIR-ATR spectroscopy. Food Chem. 2002;78: 261-266.
89. Najafi NM, Hamid AS, Afshin RK. Determination of caffeine in black tea leaves by Fourier transform infrared spectrometry using multiple linear regression. Microchem J. 2003;75:151-158.

90. Hollis DP. Quantitative analysis of aspirin, phenacetin, and caffeine mixtures by nuclear magnetic resonance spectrometry. Anal Chem. 1963;35:1682-1684.
91. Zuo Y, Chen H, Deng Y. Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and pu-erh teas using HPLC with a photodiode array detector. Talanta. 2002;57:307-316.
92. Mumin A, Akhter KF, Abedin Z, Hossain Z. Determination and characterization of caffeine in tea, coffee and soft drinks by solid phase extraction and high performance liquid chromatography (SPE-HPLC). Malaysian J Chem. 2006;8:45-51.
93. Rodrigues CI, Marta L, Maia R, Miranda M, Ribeirinho M, Máguas C. Application of solid–phase extraction to brewed coffee caffeine and organic acid determination by UV/HPLC. J Food Compos Anal. 2007;20:440-448.
94. Aklilu M, Tessema M, Redi-Abshiro M. Indirect voltammetric determination of caffeine content in coffee using 1, 4-benzoquinone modified carbon paste electrode. Talanta. 2008;76: 742-746.
95. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2(R1), International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, Switzerland, 2005.
96. Moffat AC, Osselton MD, Widdop B. Clarke's Analysis of Drugs and Poisons, 4th ed., Pharmaceutical Press, London, UK, 2011.
97. British Pharmacopoeia. The Stationary Office, British Pharmacopoeia Commission Office, London, UK, 2016; Electronic version.
98. United States Pharmacopeia 35 / National Formulary 30. Monograph on Amlodipine besylate, United States Pharmacopeial Convention, Inc., Rockville, MD, 2016; Electronic version.
99. Lange NA. Handbook of Chemistry, McGraw-Hill, New York, USA, 1961; p. 1423.
100. Carstensen JT. Kinetics pH profiles, In: Carstensen JT, Rhodes CT. Drug Stability Principles and Practices, 3rd ed., Marcel Dekker, New York, USA, 2000.
101. Dinc E. The spectrophotometric multicomponent analysis of a ternary mixture of ascorbic acid, acetylsalicylic acid and paracetamol by the double divisor-ratio spectra derivative and ratio spectra-zero crossing methods. Talanta. 1999;48:1145-1157.
102. Rao BM, Chakraborty A, Srinivasu MK, Devi ML, Kumar PR, Chandrasekhar KB, Srinivasan AK, Prasad AS, Ramanatham J. A stability-indicating HPLC assay method for docetaxel. J Pharm Biomed Anal. 2006;41: 676-681.
103. Higuchi T, Zuck DA. Investigation of some complexes formed in solution by caffeine: interactions between caffeine and aspirin, p-hydroxybenzoic acid, m-hydroxybenzoic acid, salicylic acid, salicylate ion, and butyl paraben. J Am Pharm Assoc. 1953;42:138-145.