DERIVATIVES OF THE RATIO SPECTRA FOR DETERMINATION OF ACETYLSALICYLIC ACID, ACETAMINOPHEN, AND CAFFEINE IN FIXED-DOSE COMBINATION FORMULATIONS: APPLICATION TO DISSOLUTION STUDIES

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ABSTRACT

Objective: To develop a ratio-derivative spectrophotometric method for the simultaneous quantification of acetilsalycilic acid (ASA), acetaminophen (ACE), and caffeine (CAF) in fixed-dose combination formulations. The proposed method was applied to the reference drug product Excedrin® in dissolution studies.

Methods: The method is based on the use of the first-and second-derivatives of the ratio spectra and measurements at zero-crossing wavelengths. The dissolution profiles of ASA, ACE, and CAF were obtained following pharmacopeial conditions, USP Apparatus 2 at 100 rpm and 900 ml of water. Dissolution samples were treated with the proposed UV-derivative method and the results were compared with those obtained with a validated HPLC procedure. The dissolution efficiency was used to compare dissolution profiles (HPLC *vs*. UV-derivative method).

Results: The method was linear in the range of 5-25 µg/ml of ASA, 2.5-20 µg/ml of ACE, and 1-8 µg/ml of CAF ($R^2>0.999$, *P<0.05). The precision and accuracy of synthetic mixtures were within acceptable criteria (2.11-3.43% and 96.78-104.15%, respectively). Nitrocellulose filters were the best option to filter samples and stability of all drugs was adequate when standard solutions were stored at 4 °C during 24 h. No significant differences were found between dissolution profiles (*P>0.05).

Conclusion: The proposed UV-derivative method allows the simultaneous determination of ASA, ACE, and CAF in commercial formulations. The method is simple, accurate, and precise and can be used in dissolution studies. Spectrophotometric methods are of low cost and harmless to the environment and, therefore, a better alternative than chromatographic methods.

Keywords: Acetylsalicylic acid, Acetaminophen, Caffeine, Ratio-derivative spectroscopy, Dissolution studies

Acetylsalicylic acid (ASA), acetaminophen (ACE), and caffeine (CAF) are often available in fixed-dose combination formulations. ASA possesses antirheumatic, antipyretic, and analgesic properties and is probably the major consumed drug in the world. ACE, also known as paracetamol, is an antipyretic and analgesic drug, which in contrast with ASA, it has the advantage of not irritating the gastrointestinal mucosa. The effect of both drugs on pain relief can be enhanced by CAF [1].

Taking advantage of the pharmacological properties of ASA and ACE, as well as the synergism caused by CAF, the mixture of these three drugs is used to treat migraine, among other diseases. Migraine is an episodic headache disorder that produces a wide spectrum of pain and associated disability. Migraine attacks include both pain and associated symptoms (nausea, photophobia, and phonophobia) that have a substantial impact on daily functioning. Migraine pain is typically unilateral, pulsating, and often aggravated by routine physical activity [2]. Both environmental and genetic factors play a role in the development of migraine with more than two-thirds cases having familial history and boys are more affected than girls before puberty and women are more affected than men as age increases [3].

By the wide use of this fixed-dose combination formulation generic drug products with ASA, ACE, and CAF are available in the Mexican market. Generic drug products are off-patent formulations that contain the same active pharmaceutical ingredient (API) in the same dose as the reference drug product and they are administered by the same route [4]. The interchangeability of generic drugs is understood to mean the possibility of their mutual replacement or replacement of the original drug in clinical practice [5]. Drug products containing ACE are purchased without a prescription with the potential risk of poisoning due to their inappropriate administration. Besides, the primary degradation product of ACE is *p*-aminophenol that is reported to have teratogenic effects [6].

Bioequivalence studies are the best way to assure that a generic drug product is safe and interchangeable. Before carrying out the *in vivo* studies, dissolution studies are the basic tool to evaluate the *in vitro* release performance of drugs, especially in semi-solid or solid oral dosage forms. Both studies are conducted by international regulations based on previously published scientific information [7].

Literature and experimental data indicate that ASA is a highly soluble and highly permeable drug, leading to the assignment of this drug to class I of the Biopharmaceutical Classification System (BCS) [8]. On the other hand, by its high solubility and low permeability ACE is a class III drug [9]. Immediate-release solid oral dosage forms containing ASA or ACE, as the only API, are candidates to waiver *in vivo* studies [8, 9]. In biowaiver monographs the risk of assessing bioequivalence for a specific API, based on *in vitro* rather than *in vivo* studies, is evaluated under consideration of its biopharmaceutical and clinical properties [8].

The official *in vitro* dissolution test of fixed-dose combination formulations of ASA, ACE, and CAF is described in the USP [10]. The following conditions must be used: USP Apparatus 2 (paddle) at 100 rpm and 900 ml of water as a dissolution medium (*Q* not less than 75% of each drug at 60 min). Chromatographic determination is the recommended procedure for the quantification of all drugs.

Several authors have summarized different analytical methods for the simultaneous determination of ASA, ACE, and CAF in synthetic mixtures or fixed-dose combination formulations [11, 12]. Chromatographic methods are the most accessible option but they require expensive equipment and generate toxic waste. Electrochemical methods have the advantage of presenting more sensibility, but electrochemical workstations as potentiostat/ galvanostat instruments are not widely available in pharmaceutical laboratories. UV spectroscopic methods are also proposed for

treatment of drug mixtures [13, 14]. A couple of articles describe double divisor-ratio spectra derivative methods for the analysis of the ternary mixture of ASA, ACE, and CAF. For the treatment of samples, one of this methods uses solutions of pH 11 [15], a medium that is out of physiological pH range and the other, that was specifically proposed for dissolution studies, has a confusing procedure [16].

In this study, a ratio-derivative spectrophotometric method with measurements at zero-crossing wavelengths is proposed for the simultaneous determination of ASA, ACE, and CAF in fixed-dose combination formulations. The method was developed according a previous UV analysis of a different ternary mixture [17] and the proposed UV-derivative method was applied to the treatment of dissolution samples. Results were compared with those obtained with a chromatographic method.

The ASA, ACE, and CAF standards were purchased from Sigma-Aldrich Co. (St. Louis MO, USA). The sodium phosphate monobasic and dibasic crystals, as well as methanol HPLC grade and acetic acid, were purchased from J. T. Baker-Mexico (Xalostoc-Mexico). The fixed-dose combination formulation containing ASA, ACE, and CAF (250 mg/250 mg/65 mg, respectively) used was Excedrin® tablets (GlaxoSmithKline Consumer Healthcare Mexico, S. de R. L. de C. V., Mexico). Mexican health authorities have established this commercial brand as the reference drug product to be used in dissolution and bioequivalence studies [18]. Content uniformity and assay tests were performed with the reference drug product according to pharmacopeial conditions [10].

Dissolution profiles of ASA, ACE, and CAF were obtained according to pharmacopeial conditions [10]. The USP Apparatus 2 (paddle) at 100 rpm was used (Sotax AT-7 Smart, Switzerland). Tablets were added on 900 ml of water at 37.0±0.5 °C as dissolution medium. Each dissolution profile was determined with 12 replicates. After addition of tablets 5 ml of filtered dissolution samples were withdrawn at 5, 10, 15, 30, 45, and 60 min (for HPLC analysis) and 10, 20, 30, 45, and 60 min (for UV-derivative determination). To compare dissolution profiles (HPLC *vs.* UV-derivative method) the model-independent parameter dissolution efficiency (DE) was calculated and statistically compared using a Student's *t*-test [19]. Significant differences were found if *P<0.05.

For chromatographic analysis, a Knauer HPLC equipment with UV detector and Smartline 3950 autosampler was used. A Knauer Eurospher II C$_{18}$ column (5 µm, 150 × 4.6 mm) was used. As mobile phase a mixture of water: methanol: acetic acid (65:32:3 v/v/v), previously filtered and degased, was used (1.8 ml/min). Automatic samples of 30 µl were inyected into the HPLC and drugs were quantified at 275 nm.

For spectrophotometric analysis, a double beam UV/Vis spectrophotometer (Perkin Elmer Lambda 35, Waltham MA, USA) with 1-cm quartz cells was used. The operating conditions for UV-derivative analysis were first-derivative (^{1}D) or second-derivative (^{2}D) mode with scan speed of 240 nm/min, slit width 2.0 nm, and sampling interval 1.0 nm.

The preparation of standard calibration curves of ASA, ACE and CAF were as follows: 10 mg of each drug were separately added to three 10 ml volumetric flasks. A volumen of 5 ml of methanol was added to ASA and CAF flasks and a volumen of 5 ml of 0.1 M phosphate buffer pH 7.4 was added to ACE flask; then, all flasks were sonicated during 10 min. After this, volumetric flasks were diluted to the mark with 0.1 M phosphate buffer pH 7.4. From all stock solutions five solutions of each drug, in 0.1 M phosphate buffer pH 7.4, were

prepared at determined interval concentrations: 5 to 25 µg/ml of ASA, 2.5 to 20 µg/ml of ACE, and 1 to 8 µg/ml of CAF. Then, the zero-order spectra of all solutions, from 200 to 350 nm using 1-cm quartz cells, were recorded and stored. To quantify ASA and CAF, the stored spectra of the standard solutions of ASA and CAF were divided, wavelength by wavelength and by computer aid, by the stored zero-order spectrum of a solution of 10 µg/ml of ACE. Finally, the ^{1}D of the resulting ratio spectra of ASA and CAF were plotted. The zero-crossing points to quantify ASA and CAF were identified at 244.64 and 254.89 nm, respectively. To quantify ACE, the stored spectra of the standard solutions of ASA and ACE were divided by the stored zero-order spectrum of a solution of 4 µg/ml of CAF. Finally, the ^{2}D of the resulting ratio spectra of ASA and ACE were plotted. The zero-crossing point to quantify ACE was identified at 219.17 nm. At these wavelengths all analytical signals were proportional to the concentrations of the drugs.

To quantify ASA, ACE, and CAF in dissolution samples, the zero-order spectra of filtered solutions, at adequate concentrations, were recorded and stored. Then, the stored spectra of the samples were divided by the stored zero-order spectra of 10 µg/ml of ACE and 4 µg/ml of CAF and ASA, ACE, and CAF were quantified according the procedures described above.

To test linearity, three standard calibration curves of each drug were prepared and mean data were plotted. Data were fitted by linear regression analysis and the correlation coefficients and regression analysis of variance were calculated. Precision was demonstrated with the calculation of relative standard deviation (RSD): [(standard deviation/mean) × 100] of response factor (proporcionality of response *vs.* drug concentration).

The accuracy and precision were tested with the preparation of three synthetic mixtures in 0.1 M phosphate buffer pH 7.4 from the stock solutions of each drug at following concentrations: 6, 18, and 23 µg/ml of ASA; 3, 12, and 18 µg/ml of ACE; and 2.5, 5, and 7 µg/ml of CAF. Synthetic mixtures were analyzed with the proposed UV-derivative method. Added *vs.* recovered concentrations were plotted and linear regression analysis was calculated. RDS at each level was calculated.

The drug retention by the filter was evaluated considering response of ASA, ACE, and CAF before and after a solution of each drug was filtered. Nitrocellulose and fiberglass filters were tested. Absolute differences (AD): [((initial–final)/initial) × 100] were calculated with 10 samples. The drug stability was evaluated by stored a solution of each drug at 4 and 25 °C during 24 and 48 h. The AD was calculated at each temperature and sampling time.

The zero-order spectra of a 0.1 M phosphate buffer pH 7.4 solution of 15 µg/ml of ASA, 10 µg/ml of ACE, 4 µg/ml of CAF, and a synthetic mixture of drugs at same concentrations, are shown in fig. 1A. These concentrations were used as an example of the response of the compounds to the incidence of UV light and represent the central concentration of the standard calibration curves. The zero-order spectrum of the mixture solution demonstrated a marked overlapping so that the direct determination of each drug in this solution was not possible. The zero-crossing point of CAF was found at 244.64 nm. At this point, the ^{1}D of ASA solutions were determined. The zero-crossing point of ASA was found at 254.89 nm. At this point, the ^{1}D of CAF solutions were determined. Standard calibration curves of ASA and CAF, as well as the synthetic mixture, are shown in fig. 1B. In a ^{2}D plot, the zero-crossing point of ASA was found at 219.17 nm. At this point, the ^{2}D of ACE solutions were determined. Standard calibration curve of ACE and the synthetic mixture are shown in fig. 1C.

Table 1: Linearity of acetylsalicylic acid (ASA), acetaminophen (ACE), and caffeine (CAF)

Drug	Equation	CI$_{95\%}$ for intercept	RSD (%)
ASA	y = 0.0105x – 0.0016	– 0.0053 to 0.0021	2.80
ACE	y = 0.2837x – 0.0021	– 0.0437 to 0.0396	2.54
CAF	y = –0.061x – 0.0049	– 0.0126 to 0.0027	3.16

n = 3
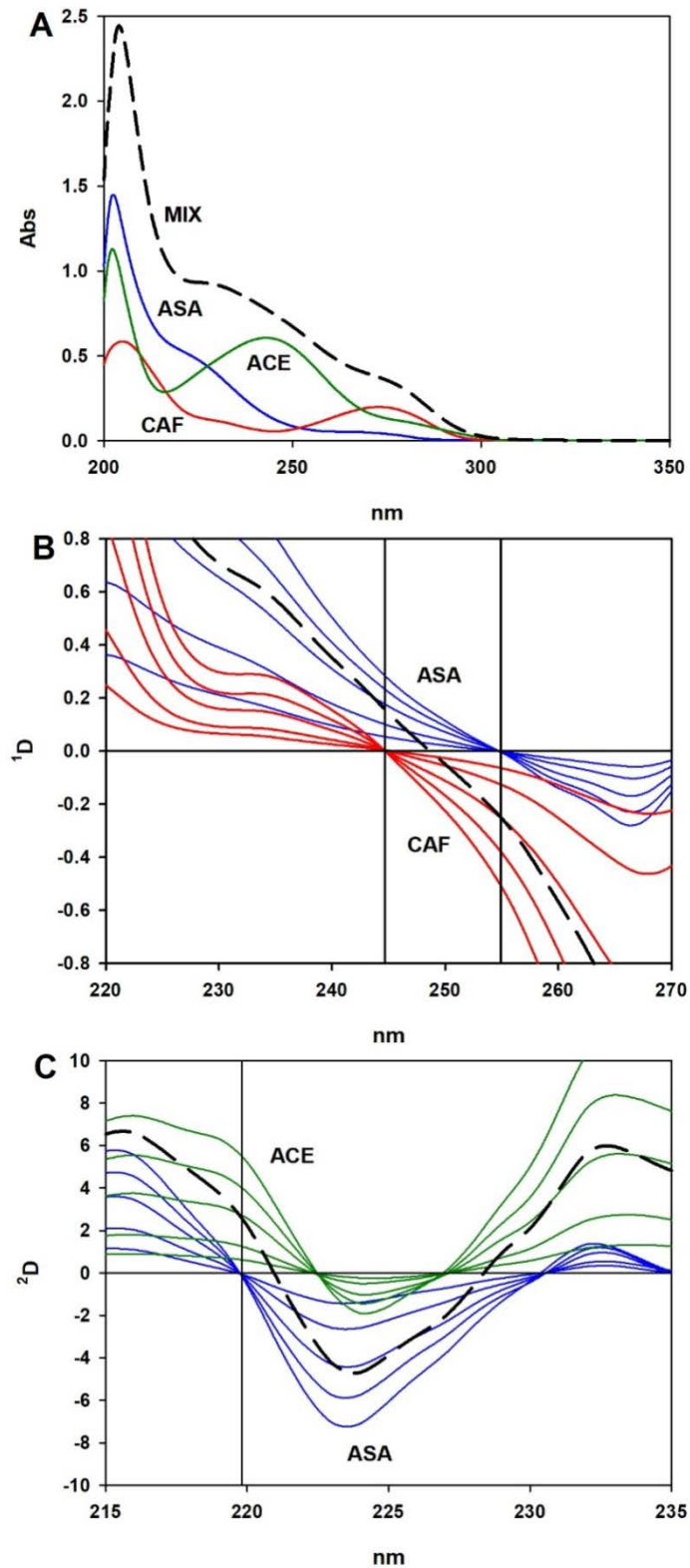
Fig. 1: A) Zero-order spectra of a solution of acetylsalicylic acid (ASA), acetaminophen (ACE), caffeine (CAF), and a synthetic mixture (MIX) with all drugs. B) First derivative (^{1}D) of the ratio spectra of standard curves of ASA, CAF, and MIX. C) Second derivative (^{2}D) of the ratio spectra of standard curves of ASA, ACE, and MIX. Vertical lines show the zero-crossing points used to quantify each drug

To test linearity, three standard calibration curves of each drug were prepared and mean data are shown in table 1. To test accuracy and precision, added *vs*. recovered concentrations were plotted and linear regressions were calculated. Results are shown in table 2. All linear regressions were significant ($R^2 > 0.999$, *P < 0.05).

Table 2: Accuracy and precision of acetylsalicylic acid (ASA), acetaminophen (ACE), and caffeine (CAF)

Drug	Equation	$CI_{95\%}$ for slope	$CI_{95\%}$ for intercept	RSD (%)
ASA	y = 0.9901x − 0.2745	0.74 to 1.23	−4.56 to 4.01	2.55
ACE	y = 1.0279x+0.0763	0.95 to 1.10	−0.85 to 1.04	2.11
CAF	y = 1.0629x − 0.2065	0.99 to 1.13	−0.60 to 0.19	3.43

n = 4

The lowest values of AD to test the influence of the filter and drug stability are shown in table 3. The reference drug product met the content uniformity and assay tests described in the USP. Results are also shown in table 3.

Table 3: AD values calculated to test influence of filter and stability with standard solutions and pharmacopeial tests to evaluate quality of reference drug product

Drug	Influence of filter (%)[a]	Stability (%)[b]	Content uniformity (min-max%)[c]	Assay (%)[d]
ASA	− 0.47	− 16.42	94.40 to 112.65	95.73
ACE	0.26	− 16.21	92.06 to 99.94	99.81
CAF	− 0.70	− 50.66	96.30 to 112.62	98.88

[a]n = 10 with nitrocellulose filters, [b]n = 6 at 4 °C during 24 h, [c]n = 10, [d]n = 3

Dissolution profiles of ASA, ACE, and CAF are shown in fig. 2. Dissolution performance of each drug, determined with the proposed UV-derivative method, looks like dissolution performance obtained with the HPLC method. As different sampling times were used in each experiment as well as at 5 min more than 80% of drug dissolved was found, no f_2 similarity factors were calculated. When the model-independent parameter DE was used to compare dissolution profiles (HPLC *vs.* UV-derivative method) no significant differences were found (*P>0.05). Results are shown in table 4.
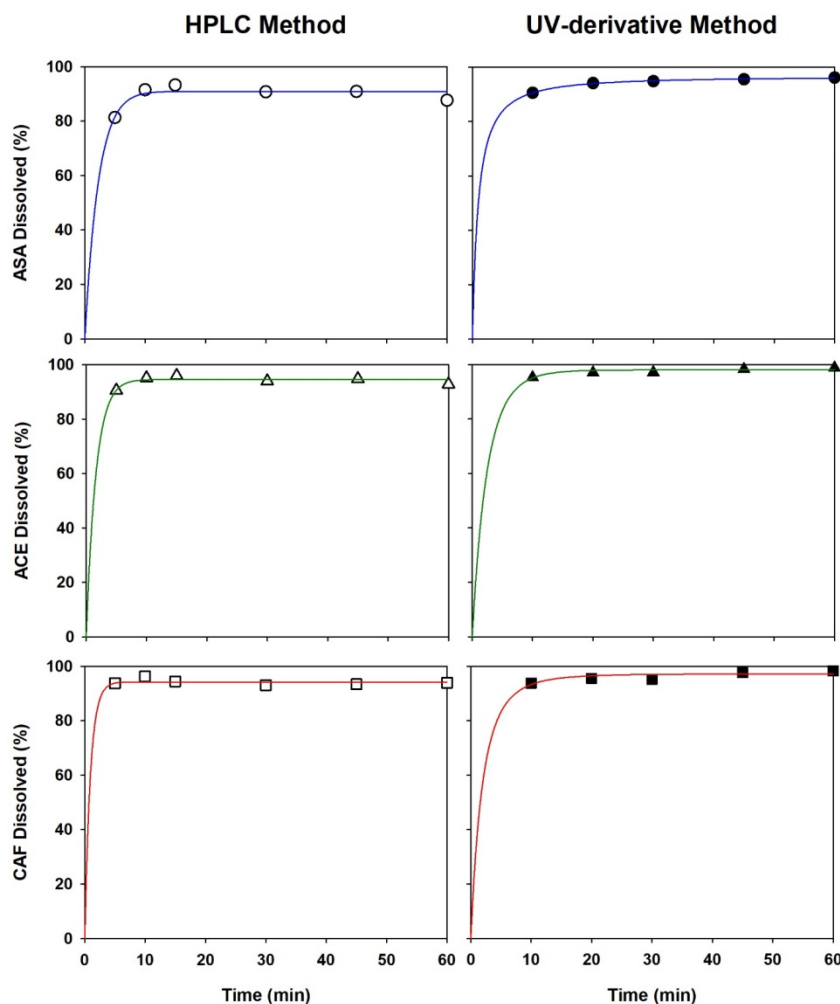
Fig. 2: Dissolution profiles of acetylsalicylic acid (ASA), acetaminophen (ACE), and caffeine (CAF) using the HPLC and UV-derivative methods, mean value, n = 12

Table 4: Dissolution efficiency values calculated with dissolution data of acetylsalicylic acid (ASA), acetaminophen (ACE), and caffeine (CAF)

Drug	HPLC method	UV-derivative method
ASA	86.31±0.59	86.37±0.62
ACE	90.26±0.48	89.19±0.32
CAF	89.95±0.33	88.04±1.08

Mean value±standard error medium. n = 12, *P>0.05

The *in vitro* release performance of ASA, ACE, and CAF was the same when HPLC and UV-derivative methods were used. The proposed spectrophotometric method could be applied with great success for the simultaneous determination of these drugs in fixed-dose combination formulations. Several authors have suggested the use of DE for the comparison of dissolution profiles [19], while this model-independent parameter is also used to establish level C *in vitro/in vivo* correlations [20].

The dissolution behavior of all drugs was fast (>80% dissolved at 5 min) so this process could not be the limited-step for the absorption of ASA, ACE, and CAF in the body. By these results the reference drug product is a good parameter to compare generic formulations. There are some considerations for requesting a biowaiver for a fixed-dose combination containing a mixture of class I and III drugs. BCS-based biowaivers are aplicable for immediate-release fixed-dose combination products if they fulfill some excipients considerations e. g. the test product contains the same excipients as the reference drug product. This is due to the concern that excipients can have a greater impact on the absorption of low permeability drugs. The composition of the test product must be qualitatively the same (except for a different color, flavor, or preservative that could not affect the bioavailability) and should be quantitatively very similar to the reference drug product [7].

Several authors have pointed out that owing to the therapeutic use of this drug combination, it is highly needed to develop fast, simple, and reliable methods for simultaneous drug monitoring in pharmaceutical formulations [11]. Ratio-derivative spectroscopy was an analytical approach successfully used to simultaneously identify ASA, ACE, and CAF in the reference drug product. Derivative methods avoid the use of toxic solvents and expensive equipment requering specialized maintenance as a HPLC apparatus. This method is an easy analitical procedure by not needing any additional mathematical calculations or working with dissolution media out of the physiological pH range. The proposed spectrophotometric method can be used to quantify these drugs in dissolution studies.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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