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Effect of internal standard on HPLC analysis of tablet dosage forms: An experimental study with statistical comparison

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ABSTRACT

The purpose of this study was to investigate the effect of internal standard on precision and accuracy of an HPLC method for the analysis of tablet dosage forms. Paracetamol was chosen as a model molecule and caffeine was used as an internal standard for this purpose. An experimental procedure was applied and a statistical comparison was performed to present the effect of IS on precision and accuracy for the quality control analysis. IS was added in a constant amount (5, 20 and 40 µg mL^{-1}) into the samples and calibration curve and internal standard techniques were used to determine the amount of PA in tablet dosage forms. As it is known, using IS corrects the loss of analyte during sample preparation or sample inlet. The results for calibration curve and internal standard techniques were statistically evaluated and discussed. According to the results in our experimental conditions, the internal standard technique statistically affected the analysis results but did not improve the precision and accuracy.

Keywords: Internal standard, HPLC, Pharmaceuticals, Paracetamol, Quality control, Statistical comparison

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INTRODUCTION

Internal standard (IS) is a chemical substance to be used for calibration in analytical chemistry by plotting the ratio of the analyte signal to the IS signal. The chemical properties of an IS are very similar, but not identical to the chemical species of interest for the analyses. It needs to provide a signal that is similar to the analyte signal in most ways but sufficiently different so that the two signals are readily distinguishable by the instrument. IS is added in a constant amount into the samples and calibration standards. This process corrects the loss of analyte during sample preparation or sample inlet. The relative amount of the analyzed compound and IS are same on samples effected by sample preparation, dilution factor or injection volume. This ratio for the samples is then used to obtain their analyte concentrations from a calibration curve [1].

Analysis of active drug compounds in pharmaceutical formulations is the routine process in quality control laboratories and it is important to use precise and accurate analytical techniques to perform the analyses [2]. A pharmaceutical company usually has to measure a large number of quality control samples. HPLC is used in the pharmaceutical industry for a wide variety of samples. Quality control/assurance of final drug products performs frequently by using HPLC [3]. The developed HPLC methods for quality control aspect has to cover the standards suggested by ICH Harmonised Tripartite Guideline [4]. When creating a new method for quantitation, the choice of the correct IS can improve the accuracy and precision of the method [5]. Therefore, the method optimization process also focuses on choosing the “best IS” to prevent the precision and accuracy of the analysis results.

Dilution variations are affected by micropipette measurement errors, and these errors are divided in two parts: systematic and random errors. Micropipette is one of the most important tools in laboratories and pipetting in the microliter range is now a current and necessary task for HPLC analysis. Precision of micropipette measurements depends only on the distribution of random errors and is not related to the true value or the specified values.

Although some of the commercial micropipettes do not conform the related standard, most of them provide the negligible precision limits [6].

Injection repeatability is related with the relative deviation for the same amount of each injection. The peak area is measured for a set of injections of the same solution. The obtained relative standard deviations (RSD %) of injections depends on how well the injector on the HPLC system can reproducibly inject the solution. Modern HPLC injectors are capable of very good injection repeatability and the precision is less than 1% [7]. Therefore, proper injector repeatability refers to the less amount of sample loss on sample inlet.

If there is no extraction or complex sample preparation processes for the analyses, the peak area of the active pharmaceutical compound will be affected by dilution variations and variations on sample inlet. It is possible to prevent the random variations on analysis by using the micropipettes meeting the standards and an HPLC method covering the required system suitability parameters [8]. At this point, it must be discussed whether using IS significantly improve the precision and accuracy of the HPLC method on the analysis of pharmaceutical formulations, or not.

In this study, a brief experimental procedure was applied and a statistical comparison was performed to understand the effect of IS on precision and accuracy for the quality control analysis in pharmaceutical industry. A well-known active pharmaceutical ingredient, paracetamol (PA) (Figure 1), was chosen as a model molecule for this purpose. An HPLC method meeting the general required system suitability parameters [8] [capacity factor (k') > 2, repeatability < 1%, tailing factor (*T*) < 2, theoretical plates (N) > 2000] for determination of PA was developed and applied for the tablet analysis. Caffeine was used as IS for this purpose. The concentration of IS were varied within three ranges (5, 20 and 40 µg mL^{-1}) and the peak area ratios of PA to IS were calculated. The analysis were performed by using the internal standard methods and calibration curve method. The statistical comparison of the analysis results were compared within each other.

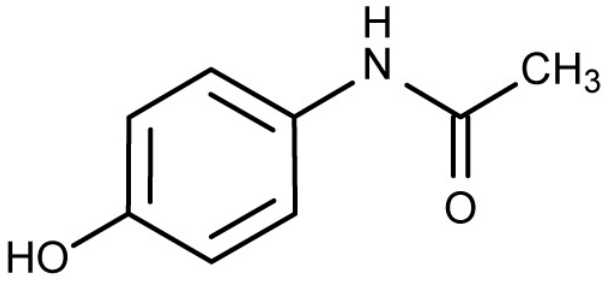

Figure 1. Chemical structure of PA

RESULTS AND DISCUSSION

According to the literature, there are lots of reported studies about the HPLC determination of PA in tablet dosage forms [9-17] and in some traditional medicines [18-26]. Some of these references and results are tabulated in Table 1.

As seen in Table 1, an IS was just used in one reported study [17]. However, the recoveries from tablet dosage forms are satisfactory and within the acceptable criteria (< 5) in general. The problem is that the experimental conditions in these studies were totally different and the variations on stationary phases, mobile phases, flow rates, and detection wavelengths could not be evaluated how the effect of IS could improve the precision and accuracy of the reported methods. The aim in the present study was to investigate the effect of IS on analysis results and the precision and accuracy of an HPLC method. Since PA is a well-known compound and lots of studies have been published for the HPLC analysis of PA in pharmaceutical dosage forms, it was used as a model molecule. HPLC conditions were evaluated according to the literature and a C18 column was used to separate PA and IS. Mobile phase was chosen as Methanol (MeOH):Water 50:50 (v/v) mixture where PA and IS eluted within 3.45 and 4.18 min, respectively. The flow rate was set at 1 mL min^{-1} and injection volume was 20 µL. The calibration and sample sets used in this experiment are given in Table 2. The chromatograms taken under experimental conditions are given in Figure 2 for sample solutions.

Table 1. An overview of the PA analysis by using HPLC

References*	Chromatographic Conditions	Internal Standard	Linearity Range	Recovery From Tablet Dosage Forms
[9]	**Column:** C18, 20cm x 4.6mm column, **Mobile phase:** 0.01M Sodium Butane sulphate in a mixture of 85 volume of water, 15 volume of methanol and 0.4 volume of formic acid, **Flow rate:** 0.2 mL min^{-1} **Injection volume:** 20 µL **Detection wavelength:** 272 nm wavelength	-	Not indicated	102.86 %
[10]	**Column:** C18 column, **Mobile phase:** MeOH:Water (65:35 v/v), **Flow rate:** 1.0 mL min^{-1} **Injection volume:** 20 µL **Detection wavelength:** 243 nm wavelength	-	5 – 50 µg mL^{-1}, R^2 = 0.995	96.52 % - 103.47%
[11]	**Column:** Shimadzu Shim-pack C18, **Mobile phase:** MeOH: 0.1% sodium acetate adjusted to pH 3.5 with acetic acid glacial(30:70 v/v) **Flow rate:** 1.0 mL min^{-1} **Injection volume:** 20 µL **Detection wavelength:** 272 nm wavelength	-	6.5-161.5 µg mL^{-1}	99.8 % - 100.1%
[12]	**Column:** C8 Column, **Mobile phase:** MeOH:0.04 M KH_2PO_4 adjusted to pH 6.0 with phosphoric acid (15:85 v/v) **Flow rate:** 1.2 mL min^{-1}	-	Not indicated	97.03% - 100.57%
[15]	**Column:** HypersilC18 (200 mm × 4.6 mm, 5µm), **Mobile phase:** MeOH:0.1 M KH_2PO_4 adjusted to pH 3.5 with phosphoric acid (70:30 v/v) **Detection wavelength:** 254 nm wavelength	-	42.88-128.7 µg mL^{-1}, r=0.9998	101.6 %
[17]	**Column:** Waters C18(300 mm × 3.9 mm, 10 µm), **Mobile phase:** MeOH:Water (1:2 v/v) **Flow rate:** 1.78 mL min^{-1} **Injection volume:** 20 µL **Detection wavelength:** 193 nm wavelength	Sulphamethoxalone	10.0 – 30.0 µg mL^{-1}	99.2% - 99.3%

** Chromatographic conditions and experimental results are taken from the manuscripts or abstracts as they are indicated.*

Table 2. Calibration and sample sets used in the experiments

Calibration Set 1		**Calibration Set 2**		**Calibration Set 3**	
Concentration*		**Concentration***		**Concentration***	
PA	**IS**	**PA**	**IS**	**PA**	**IS**
1	40	1	20	1	5
5	40	5	20	5	5
10	40	10	20	10	5
15	40	15	20	15	5
20	40	20	20	20	5
30	40	30	20	30	5
Sample Set 1a		**Sample Set 2a**		**Sample Set 3a**	
Concentration*		**Concentration***		**Concentration***	
PA	IS	PA	IS	PA	IS
5	40	5	20	5	5
Sample Set 1b		**Sample Set 2b**		**Sample Set 3b**	
Concentration*		**Concentration***		**Concentration***	
PA	IS	PA	IS	PA	IS
20	40	20	20	20	5

** Concentration values are given as µg mL^{-1}*

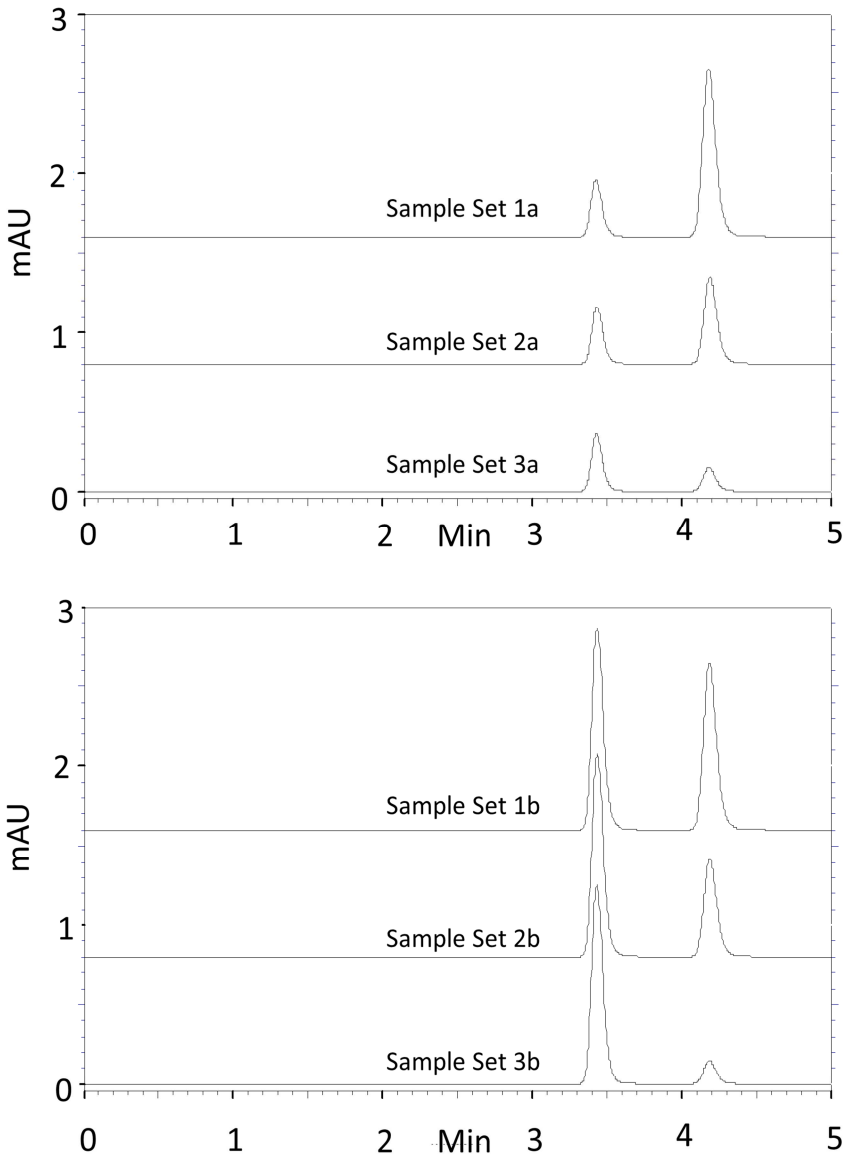

Figure 2. Overlaid chromatograms of sample sets taken under experimental conditions

Initial experiments show that the peak areas of PA were increased linear up to 30 µg mL^{-1}. The limit of detection for PA was 0.1 µg mL^{-1}. The PA concentrations were between 1.0 and 30.0 µg mL^{-1}, and IS concentrations were 5, 20 and 40 µg mL^{-1} for the calibration sets. The regression equations for calibration sets (Table 2) are given in Table 3.

Table 3. The regression equations for calibration sets

	Concentration		y= ax + b (where x: concentration µg mL^{-1})	
Calibration Set	**PA (µg mL^{-1})**	**IS (µg mL^{-1})**	**Calibration Curve* y1 : peak area of PA**	**Internal Standard* y2: peak area ratio of PA to IS**
1	1 – 30	40	a: 370727 ± 489 b: 433833 ± 11416 RSD of a: 0.32 RSD of b: 6.45	a: 0.05483 ± 0.00025 b: 0.00563 ± 0.00252 RSD of a: 0.10 RSD of b: 10.94
2	1 – 30	20	a: 372189 ± 8425 b: 408076 ± 29062 RSD of a: 5.54 RSD of b: 17.44	a: 0.09932 ± 0.00283 b: 0.13588 ± 0.00839 RSD of a: 6.98 RSD of b: 15.13
3	1 – 30	5	a: 373054 ± 1845 b: 220020 ± 7829 RSD of a: 1.21 RSD of b: 8.71	a: 0.35404 ± 0.00648 b: 0.33755 ± 0.01402 RSD of a: 4.48 RSD of b: 10.17

** RSD: Relative standard deviation*

The variations on IS concentrations are significant if they effect the regression equation on internal standard technique. The standard errors on the intercepts demonstrate that the method has a significant non-specific bias. In such conditions, low amount of samples are hard to be analyzed. As it is seen from Table 3, the internal standard technique did not have a significant impact on the standard errors of slope and intercept. The random or systematic errors affecting the calibration curve techniques were identical on the internal standard techniques. All the calibration sets were prepared by same analyst and the results on calibration curve techniques for set 1, 2 and 3 should be identical theoretically if there were no errors. However, the deviations on slope and intercept of calibration curve techniques for sets were also seen on internal standard techniques. This situation might express that the same errors on preparing standard solutions appear clearly on internal standard techniques and IS did not help to improve the deviations in our experimental conditions, or it cannot be statistically proved.

In the present study, three different calibration sets given in Table 2 were used to calculate the two different amount of PA in samples (5 and 20 µg mL^{-1}). The PA concentrations in the sample sets (Table 2) calculated by calibration curve and internal standard techniques are summarized in Table 4.

Table 4. Analysis results of sample sets by using calibration curve and internal standard techniques

	Sample Set 1a*		Sample Set 2a*		Sample Set 3a*	
	Concentrations PA=5 µg mL^{-1}, IS=40 µg mL^{-1}		Concentrations PA=5 µg mL^{-1}, IS=20 µg mL^{-1}		Concentrations PA=5 µg mL^{-1}, IS=5 µg mL^{-1}	
Parameter**	Calibration Curve	Internal Standard	Calibration Curve	Internal Standard	Calibration Curve	Internal Standard
Mean	4.836	4.912	4.774	4.945	4.990	5.230
SD	0.019	0.069	0.077	0.154	0.084	0.122
SE	0.008	0.028	0.032	0.063	0.034	0.050
RSD %	0.385	1.399	1.619	3.122	1.683	2.341
Bias %	3.286	1.756	4.519	1.107	0.198	-4.595
	Statistical Comparison (t test)***		Statistical Comparison (t test)***		Statistical Comparison (t test)***	
p value	0.0372		0.0516		0.0048	
	Sample Set 1b*		Sample Set 2b*		Sample Set 3b*	
	Concentrations PA=20 µg mL^{-1}, IS=40 µg mL^{-1}		Concentrations PA=20 µg mL^{-1}, IS=20 µg mL^{-1}		Concentrations PA=20 µg mL^{-1}, IS=5 µg mL^{-1}	
Parameter**	Calibration Curve	Internal Standard	Calibration Curve	Internal Standard	Calibration Curve	Internal Standard
Mean	20.042	21.195	19.608	20.139	20.698	20.011
SD	0.526	0.331	0.569	0.640	0.412	0.140
SE	0.215	0.135	0.232	0.261	0.168	0.057
RSD %	2.626	1.560	2.904	3.179	1.989	0.700
Bias %	-0.209	-5.973	1.960	-0.696	-3.492	-0.053
	Statistical Comparison (t test)***		Statistical Comparison (t test)***		Statistical Comparison (t test)***	
p value	0.0020		0.1958		0.0054	

** Sample set 1a and 1b, 2a and 2b, 3a and 3b were evaluated by using calibration sets 1, 2 and 3, respectively.*
*** Mean is the average value of the PA concentration found in the sample set, SD is the Standard deviation, SE is the standard error, RSD % is the relative standard deviation, and Bias% is the accuracy of the method.*
**** Statistical comparison was performed by t-test (equal variance)*

According to the Table 4, the relative standard deviations were from 0.38 to 2.90 and 1.39 to 3.17 whereas the bias values were from -0.21 to 4.51 and -5.97 to 1.75 for calibration curve and internal standard techniques, respectively. These results indicate that the effect of internal standard on the precision and accuracy was not significant in our experimental conditions. However, the calibration curve technique gave statistically different results ($p < 0.05$) in comparison to internal standard technique except for sample set 2a and 2b. This situation might be explained if the IS concentration is low (5 µg mL^{-1}) or high (40 µg mL^{-1}) in comparison to the analyzed compound; the random errors affecting the peak area of IS also affect the analysis results. These random errors may not be significant for identical concentrations. The other interesting situation is that the internal standard statistically affects the analysis results but the effect on the results could not be directly correlated with the precision and accuracy. The relative standard deviation and bias values given in Table 4 are in a wide range and it is hard to say these results were improved by the effect of internal standard in our experimental conditions.

MATERIALS AND METHODS

Chemicals and Solutions

MeOH was analytical reagent grade from Merck (Darmstadt, Germany). Milli-Q water system (Barnstead, USA) was used for the preparation of buffer and other aqueous solutions. Standard stock solutions of PA and IS were prepared as 1000 µg mL^{-1} in MeOH.

Preparing Calibration Standards

Eppendorf Research®plus micropipettes (adjustable for maximum 100 µL and 1000 µL volumes) were used to prepare the calibration standards. Three different set of calibration standards containing between 1 and 30 µg mL^{-1} of PA and 5, 20 and 40 µg mL^{-1} of IS were prepared in the HPLC vials for six times. The solutions having concentration below 20 µg mL^{-1} for CA or IS were prepared by using the diluted standard stock solutions (100 µg mL^{-1} of CA or IS). All of the standard stock solutions were prepared in HPLC vials and the volume was filled up to 1000 µL with MeOH:Water (50:50 (v/v)) mixture. Finally 126 solutions were prepared for seven points calibration curve of these three set (n=6).

Preparation of Sample Solutions

Ten tablets (Parol® Tablets) containing 500 mg PA were weighed to determine their mean weight, and finely powdered in a mortar. An amount of powdered mass equivalent to one tablet was accurately weighed and transferred to a 100 mL volumetric flask. A 50 mL volume of MeOH was added into the volumetric flask and then sonicated for 15 min to ensure complete dissolution of PA. The flask was then filled up to volume with MeOH. An aliquot from this solution was filtered through a 0.45 µm membrane filter. The final tablet solutions contain 5000 µg mL^{-1} of PA. This procedure was repeated for six times and the final solutions were diluted 50 times with MeOH. Appropriate amount of the final sample solutions containing 100 µg mL^{-1} of PA were taken into a vial by using micropipette to prepare the sample sets containing 5 and 20 µg mL^{-1} of PA. These solutions were prepared for six times for three different IS concentrations (5, 20 and 40 µg mL^{-1} of IS). Finally 36 solutions were prepared for sample analysis having different amount of PA and IS.

Apparatus and Analytical Conditions

The LC system consisted of a Spectra-SYSTEM P2000 gradient pump, a Spectra-SYSTEM SCM 1000 degasser, an automated injector and a Spectra-SYSTEM UV2000 detector (Thermo Separation Products, USA). The detector was set at 256 nm and peak areas were integrated automatically by ChromQuest software. The experiments were performed using a reversed-phase Phenomenex C18 column (250 mm x 4.6 mm I.D.) column. The LC system was operated isocratically at room temperature using a mobile phase consisted of MeOH:Water (50:50 (v/v)) mixture. After mixing, the mobile phase was filtered through a 0.45 µm membrane filter and run at a flow rate of 1 mL min^{-1}. Injection volume for both calibration standards and samples was 20 µL. All of the analyses were carried out at ambient temperature (22 – 27 °C).

Creating the Calibration Curves and Analyzing the Samples

According to the experimental procedure given above 3 different calibration sets and 6 different sample sets were prepared for six times. The list of these solutions is given in Table 1. The calibration curves were constructed by two different ways. The first approach was to plot the concentrations to the peak areas of PA at 256 nm wavelength (Calibration curve technique). The second approach was to use each calibration set individually and plot the concentrations to the peak area ratios of PA to IS at 256 nm wavelength (Internal standard technique). The calibration sets and sample sets are given in Table 2.

Statistical Analyzes

Statistical analysis was performed by using Student's t-test on the sample sets given in Table 1. Calibration curve and internal standard techniques were used to determine the PA amount in the samples.

CONCLUSION

In this study, an experimental model, the analysis of PA while using caffeine as IS, was used to evaluate the effect of IS on the precision and accuracy of the tablet analysis results on HPLC analysis. A statistical comparison was performed on analysis results of tablet solutions containing 5 and 20 µg mL^{-1} of PA while using 5, 20 and 40 µg mL^{-1} of IS. The results on our experimental conditions shows that internal standard technique statistically affects the analysis results but do not improve the precision and accuracy of the method. This situation might be expressed with the usage of highly precise micropipettes in our experiments and high injection repeatability (< 1% RSD) of the HPLC instrument. However, it could be concluded whether internal standard would improve, or not the tablet analysis results should be evaluated in every single experimental conditions for each active pharmaceutical ingredients and pharmaceutical formulations for whom the HPLC analysis were applied by.

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