Compounds Chemically Related to Analyte as Surrogate Reference Standards in Quantitative HPLC: Preliminary Study and Proof of Hypothesis

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ABSTRACT: *Purpose:* Compounds chemically related to analyte as surrogate reference standards in quantitative HPLC and the impact of internal standard on such applications have been investigated. *Method:* A simple reversed-phase isocratic HPLC method with UV detection was developed and validated. The solutes were paracetamol (principal analyte), caffeine (internal standard) and candidate surrogate reference standards (aspirin, benzoic acid and phenacetin). The chromatographic conditions were Zorbax C-18 column, methanol/ 2.5% ethanoic acid (2:3) mobile phase and UV detection at 257nm. The relationship between signal intensities and concentrations of a pair of analyte and candidate surrogate reference standard was used to determine a constant ($S_{á}$) which was later used in a derived equation to evaluate the content of nine brands of paracetamol tablets. *Results:* The retention times of the solutes were 8.1 ± 0.03 min (aspirin), 11.7 ± 0.05 min (benzoic acid), 4.7 ± 0.02 min (caffeine), 3.0 ± 0.01 min (paracetamol) and 11.1 ± 0.06 min (phenacetin). $S_{á}$ for the various candidate surrogate reference standards were: 18.23 ± 0.048 (aspirin), 11.66 ± 0.251 (benzoic acid) and: 1.15 ± 0.051(phenacetin). The assay values with each of the candidate surrogate reference standards either met the monograph requirements of the United States Pharmacopoeia and National Formulary (2004) or the British Pharmacopoeia (2007) or both. Effect of internal standard was void. *Conclusion:* Surrogate reference standards were successfully applied to the assay of paracetamol tablets. The proposed method can potentially be used in routine quantitative HPLC applications once the HPLC method is developed and $S_{á}$ is also determined with previously available chemical reference standard.

Keywords: Surrogate reference, surrogate constant, pharmacopoeia, HPLC, analyte

INTRODUCTION

Counterfeit, adulterated and substandard medicines are a global menace. The Centre for Medicines in the Public Interest (CPMI), USA, estimates that in 2010, the global sale of counterfeit drugs will be $75 billion, a 92% increase from 2005 (1). It has also been estimated that up to 15% of all sold drugs are fake, and in parts of Africa and Asia, this figure exceeds 50% [2; 3, 4; 5, 6; 7, 8]. This is a serious and rapidly growing problem due to lack of resources to deal with the situation in developing countries as well as close cooperation between drug companies, governments, or international organizations concerned with trade, health, customs & excise and counterfeiting [9].

Efforts to safeguard the quality of medicines usually involve the application of instrumental methods for both qualitative and quantitative analyses of active pharmaceutical ingredients in bulk and formulations. The high performance liquid chromatography (HPLC) technique has been extensively used and recommended for both in vitro and in vivo quality monitoring of medicines [10; 11, 12]. However, most HPLC applications in both pharmaceutical and biomedical problems require the use of chemical reference standards for identification and/or quantitation. This usually helps in preparing controls, calibration curves and system suitability tests for analyses. This notwithstanding, accessibility, cost of reference standards (Table 1) and shipment (for varied active pharmaceutical ingredients (APIs) in multi-source drug products) often make it difficult for developing countries to fully utilize the capacity of the HPLC for in-process, finished product and regular post market quality monitoring of medicines. Sometimes, a laborious and low yielding alternative method is used to prepare

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secondary standards (working standards) from drug products without standard facilities (IR, DSC, MS and NMR) to substantially characterize the secondary standard.

Our study therefore sought to investigate the use of compounds (Figure 1) chemically related to analytes as surrogate reference standards in HPLC and the impact of internal standard on such applications. The physico-chemical properties for compound suitability as a surrogate reference standard and the correlation between results of an assay with a surrogate reference and official methods were also examined. There are reports of UV spectrophotometric assay of some medicinal compounds without necessarily using chemical reference standards. Such assays rather use the specific absorbance (A (1%, 1cm)) of the substance which has been previously determined with an authentic sample. This approach is the specification of the British Pharmacopoeia for the assay of diazepam tablets [13].

Table 1
Price Quotes for some Chemical Reference Standards from the United States Pharmacopoeial Convention, Inc., 2009

Reference Standard	*Quantity / unit*	*Price ($)*
Amoxicillin	200 mg	194.00
Clavulanate Lithium	200 mg	194.00
Ciprofloxacin	200mg	154.00
Ciprofloxacin Hydrochloride	400 mg	194.00

Other pharmacopoeias such as the United States Pharmacopoeia and National Formulary (USP&NF) and International Pharmacopoeia (IP) have similar examples of assays. The use of nuclear magnetic resonance (NMR) in quantitative analysis of some pharmaceuticals without the use of reference standards has also been reported [14]. However, there were no available records of a study involving the use of surrogate reference standards in quantitative HPLC and this study sought to contribute to filling that gap. The ultimate aim of finding alternatives to routine use of chemical reference standards in quality assessment of medicines shall make it possible for pharmaceutical industries, drug regulatory bodies and drug quality research institutions in developing countries to sparingly use CRS and conserve the limited financial resources for other research needs.

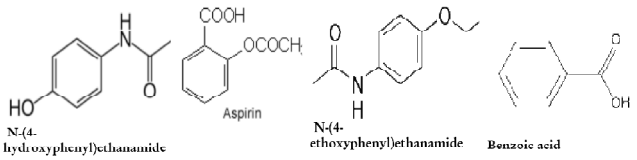

Figure 1: Chemical Structures of Study Samples

Paracetamol (*N*-(4-Hydroxyphenyl) ethanamide) tablets were chosen for this initial study because paracetamol was readily available both as a CRS and formulation. It was also possible to get all the required reagents and technical support to adequately characterize, prepare and analyse the samples. Nine different brands of tablets which were the most patronized brands in the Kumasi metropolis after an initial survey were selected. Phenacetin (*N*-(4-Ethoxyphenyl) ethanamide), benzoic acid and aspirin (2-acetoxybenzoic acid) were considered as candidates for surrogate reference standards because they were related to paracetamol in terms of solubility and UV absorption pattern for simultaneous elution and UV detection [13, 15].

STUDY HYPOTHESIS

In instrumental analysis, beyond limit of quantitation and within limits of linearity, signal intensity is directly proportional to concentration of solute. If the concentration of a given solute is $\boldsymbol{C}$ and corresponding signal intensity is $\boldsymbol{A}$, mathematically; $k = A/C$ where k is a constant of proportionality and specific for a particular substance under a given set of experimental conditions. Therefore, for two solutions of the same compound with concentrations C_s and C_t respectively, with corresponding signal intensities of A_s and A_t; $A_s/C_s = A_t/C_t$

However, if the two solutions were for different compounds that can be co-eluted isocratically with the same respective concentrations and signal intensities as above, their respective constants of proportionality k_s and k_t will be different and consequently; $A_s/C_s \neq A_t/C_t$

If it is assumed at this instance that; $A_t/C_t \alpha A_s/C_s$, then, it can be deduced that; $k_t/k_s = A_tC_s/C_tA_s$. Therefore; $A_t/C_t = (k_t/k_s)(A_s/C_s)$.

$$\text{If } k_t/k_s = S_\alpha,\ A_t/C_t = S_\alpha(A_s/C_s) \qquad (1)$$

S_α is a constant that is being reported as the surrogate constant. If A_t and A_s are the respective signal intensities of a principal analyte and a

surrogate reference standard with C_t and C_s as corresponding concentrations, then C_t which can also be referred to as the actual concentration of analyte (when S_α is known) can be determined once the other variables in Equation (1) are known. Usually in quantitative analysis, nominal concentrations are prepared from the strength of product indicated on the label and the assay value is obtained by expressing as a percentage, the ratio of the actual to nominal concentrations.

EXPERIMENTALS

Materials/ Reagents

The following chemicals were provided by the Department of Pharmaceutical Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi, Ghana: Phenacetin (BDH), o-acetylsalicylic acid (BDH) , benzoic acid (BDH), methanol (BDH), glacial acetic acid (BDH), acetic anhydride (BDH), toluene (BDH), sodium hydroxide (Fisons), hydrochloric acid (Fisons), ethanol (BDH), tetraoxosulphate (vi) acid (Fisons), ammonium cerium (IV) sulphate (BDH), sodium thiosulphate (BDH), potassium hydrogenphthalate (BDH) and perchloric acid (BDH). All the chemicals were of analytical grade. HPLC grade methanol and deoinised water were used for HPLC work. Reference paracetamol powder was obtained from the Food and Drugs Board, Ghana, while the paracetamol tablets (Table 2) were bought from retail pharmacies in Kumasi, Ghana, between March and April 2006 with each having one year or more of its shelf-life remaining except brands EF and KL which had no information on expiry date.

Table 2
Melting Range, Density, Percentage Purities and Mean Retention Time (n = 10) of Study Samples

Sample	*Melting range(° C)*	*Purity (%)*	*Mean retention time(min)*	*Density (gcm^{-3})*
Aspirin	140-142 (about 143)	97.69 (99.5–101.0)	8.05 ± 0.028	1.3500
Benzoic acid	122- 124 (121-124)	101.03 (≥95)	11.73 ± 0.054	1.3000
Caffeine	233- 235 (234-239)	103.02 (98.5–101.5)	4.72 ± 0.019	1.2300
Paracetamol	168-170 (168-172)	99.47 (99.5–101.0)	3.02 ± 0.012	1.2930
Phenacetin	133-136 (134-136)	99.77 (99.5–101.0)	11.11 ± 0.060	1.2404

Instrumentation

The liquid chromatograph consisted of Spectra System P4000 pump, Zorbax ODS column (4.6mm x 25 cm), Spectra System UV1000 detector and Dell Pentium IV with Chromoquest software for chromatograms and integration. Other equipment include Griffin Flask Shaker, Cecil 8000 Series Double Beam UV-Visible Spectrophotometer, and pH meter (Denver Instruments, UB-10).

Methods

Characterization of aspirin, benzoic acid, phenacetin and paracetamol (pure powder and tablets)

To confirm the identity, purity and content of each of the samples, the monograph requirements of BP 1980 Vol I [16] (phenacetin) and BP 2007 Vol I [13] (aspirin, benzoic acid and paracetamol) were used. The requirements mostly involved examining appearance of samples, determining solubility and melting point, performing colour reactions and volumetric assays and sometimes chromatography and UV spectroscopy. Each chemical sample was treated on its merit. The different brands of paracetamol tablets were also respectively assessed according to the monograph requirements of BP 2007 Vol II [13]. The assessments included extractions of paracetamol from tablet samples, determining melting point, performing colour reactions and following a specified assay protocol for determining the percentage contents of each of the brands. Before each assay, the uniformity of weight test as described in BP 2007 was done. This was to ensure that the set of twenty tablets that were randomly selected to define the average weight of a tablet for a brand was uniformly distributed. This was because each assay required taking a certain weight of powdered tablets equivalent to a specified amount of active ingredient, using the equivalent relationship between the average weight and nominal strength of the tablet.

Developing the isocratic HPLC method for eluting paracetamol, candidate surrogate reference and internal standards

The development of an isocratic chromatographic system that could separate, detect and quantify the selected compounds in a single run started off by considering the physico-chemical properties and UV absorption patterns (acidic, basic and neutral) of the analytes from Clarke's Analysis of Drugs and Poisons [15] and the BP [13]. Caffeine (1,3,5-trimethylxanthine) was added to the list of compounds to monitor the impact of internal standard on subsequent applications of this study. The information obtained on the selected compounds indicated their respective reasonable polarity with good UV absorption. We therefore considered a reverse-phase chromatography with UV detection. All the selected compounds were freely soluble in alcohol (90% ethanol) except caffeine which was slightly soluble. As a result, a combination of methanol and water (1:1) was found suitable for dissolving all the selected compounds into a homogenous mixture. In working up the mobile phase, a number of methanol-deoinised water combinations were tried, in each case, separation efficiency, resolution and run time were monitored. Some of the combinations were methanol: water (1:1), methanol: water: acetic acid (28:69:3) and methanol: 2.5% glacial acetic acid (2:3). Parameters that were basically varied to optimize the separation were the ratio between organic and aqueous contents, ionic strength and pH. Ionic strength and pH were modified with glacial acetic acid. The mobile phase that gave satisfactory separation, resolution and reasonably good run time for all the analytes (< 12 minutes) was methanol/2.5% glacial acetic acid (2:3). The other combinations had at least one of the following poor chromatogram characteristics: overlapping bands, tailing peaks and unduly long retention times (> 20 min). A flow rate of 1.0mL/min and UV detection at 257nm were found as optimal for separation and detection respectively.

Characterization of chromatograms and method detection limits

After obtaining chromatograms for the mixture of study samples, the various peaks in the chromatograms were identified by the respective retention times of the samples. A solution (50.0µg/mL) of each of the samples was prepared with the diluent (methanol/water (1:1)) and each solution eluted with the conditions established earlier to find the individual retention times. The minimum detection limit (MDL), the limit of detection (LOD), limit of quantitation (LOQ), signal to noise ratio (SNR) and other relevant analytical performance parameters were also evaluated. After a number of investigations, a solution containing benzoic acid (4.0µg/mL), aspirin (30.0µg/mL), paracetamol (1.5µg/mL), phenacetin (2.0µg/mL) and caffeine (20.0 µg/mL as internal standard) was prepared with the diluent and eluted. The samples were pooled together to form a common solution for each one to serve as matrix for the other at any point in time. Ten replicate determinations were made and the peak area ratios (peak area of sample/peak area of internal standard) of each compound calculated. Concentrations were then interpolated from previously constructed calibration curves for each of the compounds except caffeine. Concentrations for each compound (n= 10) were statistically analysed to obtain the mean concentration and standard deviations. These data were subsequently used to determine the respective MDL, LOD, LOQ, SNR and other relevant analytical performance parameters. We also investigated the robustness of the surrogate constant of each surrogate reference candidate to variations in concentration of principal analyte (paracetamol) by analyzing four other concentrations of paracetamol solutions (3.0-7.0 µg/mL) and calculating the surrogate constants from the data obtained. Each of the samples was dried in the oven at 100°C for 6 hrs and kept in a desiccator before preparing the solutions.

Determination of surrogate constants (S_4) and assay of paracetamol tablets using candidate and pharmacopoeial methods

A solution containing pure paracetamol, aspirin and caffeine was accurately prepared with the diluent such that the concentrations of the samples in the final solution were respectively 2.0µg/mL, 30.0 and 20.0µg/mL (Solution A). Two other solutions (B&C) were similarly prepared with each containing either benzoic acid (4.0µg/mL) or phenacetin (2.0µg/mL) instead of aspirin in solution A. Stock solutions (1.0mg/mL) of all the samples were individually prepared and appropriate volumes of each pooled together in a 100ml volumetric flask and made up to volume to constitute either solution A or B or C. Each of the solutions was eluted in ten replicates with the

chromatographic conditions earlier established, injecting 20µL manually each time. Details about peak area ratios and concentrations of pure paracetamol and surrogate references were compiled and surrogate constants for various candidates calculated from Equation (1).

With respect to the assay, a set of twenty tablets were randomly selected for each of the nine brands. Their respective average weights were taken and the tablets were powdered and stored in labeled containers in a cool dry place away from light. For each of the nine brands, weight of powdered tablets equivalent to 0.1g of paracetamol was taken. This was transferred into a 100mL volumetric flask containing about 30mL of the diluent and placed in a mechanical shaker for 10min. The solution was made to volume with the diluent and filtered with Whatmann's no. 1 filter paper, discarding the first few milliliters of filtrate. The rest of the procedure was the same as preparing and analyzing solutions A, B and C, replacing the pure paracetamol in each solution with that of the tablet samples (2.0µg/mL). The actual concentrations of the tablet samples were calculated from Equation (1) and consequently, the respective percentage contents. System suitability test was performed each day that analyses of tablets were carried out. The official methods of the BP and USP were also used without any modifications to assay each of the nine brands of tablets and the results compared statistically with the candidate method. Seven replicate determinations were made for each assay under this section.

STATISTICAL ANALYSES

Graph Pad Prism Version 5 was used for means, standard deviations, outliers, Bonferroni's multiple comparison tests and the Bartlett's test for equal variances.

RESULTS

The general results of the study have been organized and reported in Tables and Figures. Data for most parameters have been reported as mean ±sd. Table 2 shows that the melting ranges of the pure powders were characteristic according to the specifications of the British Pharmacopoeia (in parenthesis). Though the lower limits for caffeine and phenacetin were marginally less than the specified, the upper limits fell within standard ranges. They were therefore accepted as characteristic within limits of experimental error. However, the mean percentage purity of caffeine (103.02) from Table 2 also marginally exceeded the upper limit of the monograph requirement (101.5%) suggesting that, the cause of the dip in melting range might be due to an impurity. Caffeine was still maintained in the study because it was used as an internal standard, which should affect equally the peak area ratios of the other compounds. The assay value of each sample was factored into all calculations and measurements. The identity and purity of the pure powders were therefore generally acceptable according to the monograph requirements of BP 1980 and 2007 [13, 16] and suitable for use in the study.

The identity of the brands of paracetamol tablets were also confirmed according to the standards of BP 2007 [13]. Brands EF and KL appeared suspicious because they did not have batch numbers and expiry dates as shown by the double asterisk in Table 3. However, the colour identification and melting range determinations (not shown) confirmed the tablets as paracetamol. The uniformity of weight across the brands was

Table 3
Profile of Paracetamol Tablets Studied (500mg)

Brand	*Manufacturing Company*	*Batch number*	*Expiry date*	*Average weight (g)*
AB	Pharm AB Ltd	6002116	01/08	0.5962±0.019
CD	Pharm CD Ltd	035	08/09	0.5499±0.007
EF	Pharm EF Ltd	**	**	0.5644±0.004
GH	Pharm GH Ltd	PA393H	09/08	0.5762±0.008
IJ	Pharm IJ Ltd	F03013	02/07	0.5540±0.004
KL	Pharm KL Ltd	**	**	0.5698±0.009
MN	Pharm MN Ltd	75	07/08	0.6060±0.013
QR	Pharm QR Ltd	11	01/09	0.6113±0.020
ST	Pharm ST Ltd	13	01/08	0.5679±0.007

also acceptable because none of the brands deviated by more than 10%w/w of the average weight as required by BP 2007[13]. The paracetamol tablets were also authentic for the study. The tablet samples were collected in 2006 and analysed before expiry (Table 3). Tablet brand names and manufacturers were coded to conceal identity, however, the batch numbers can still be traces of identity with some effort except for brands EF and KL.

In Table 4, details about the analytical performance parameters of the study samples have been provided. Generally, all the samples produced data with sufficient reliability for evaluation of the study objectives. The MDL, LOD, LOQ and the SNR values were appropriate and supported the working concentrations used. The MDL was determined at 99% confidence level and the t-statistic for 10 replicates at p = 0.01 was 2.821.This was used for the calculation of the MDL. The values of the surrogate constants showed a variation that could be linked to the differences in the structural and inherent physico-chemical differences among the candidate surrogate reference standards. The constant is characteristic for each candidate surrogate reference under a set of experimental conditions. The numerical value, in itself, provides little information about the effectiveness of the surrogate reference standard until it is factored into Equation (1). Table 5 shows that despite the wide numerical differences among the constants, results for content evaluation of the tablets with each candidate surrogate reference met at least one of the pharmacopoeial limits. The linear relationship between concentration and signal intensity generally levels off the numerical differences among the surrogate constants when Equation (1) is used. Figure 2 demonstrated that variations in concentration of principal analyte (low, medium and high) did not produce significant change in surrogate constant for each of the candidate surrogate reference compounds. In Figure 3, the selectivity of the proposed method was evidenced by the representative chromatograms. It is clear from the chromatograms that the analytical conditions could separate and resolve reasonably the study samples.

The application of the proposed method in the assay of the tablets yielded results that were either

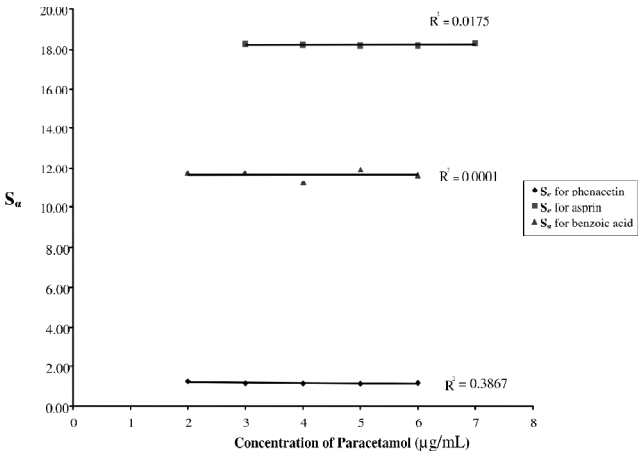

Figure 2: Variation of Surrogate Constant (S_α) with Concentration of Analyte

in compliance with the monograph requirements of the BP (95.0-105.0%w/w) or USP (90.0-110.0%w/w) or both. The results of both the proposed and official methods correlated positively (Table 5) and had comparable precisions (Table 6).

DISCUSSION

The HPLC has been used to identify and assess the purity and/or content of many pharmaceutical substances. Analytical procedures currently used in specifications for pharmaceutical substances and products that may require a chemical reference substance include methods based on chromatographic separation for identification or quantitative purposes [17]. Chemical reference standard according to the WHO Expert Committee on Specifications for Pharmaceutical Preparations refers to an authenticated uniform material that is intended for use in specified chemical and physical tests, in which its properties are compared with the properties of a product under examination, and which possesses a degree of purity adequate for its intended use [18].

According to the guidelines of the International Pharmacopoeia 2006 [17], the general use of a chemical reference substance should be considered an integral part of a compliance-oriented monograph or test procedure used to demonstrate the identity, purity and content of pharmaceutical substances and preparations. However, these substances are normally prepared and issued by regional/national pharmacopoeial commission or the regional/national quality control laboratory on behalf of drug regulatory authorities.

Table 4
Analytical Performance Parameters and Surrogate Constants (n = 10)

Sample	*Aspirin*	*Benzoic acid*	*Phenacetin*	*Paracetamol*
Parameter				
Conc.(µg/mL)	30.0	4.0	2.0	1.5
Mean Conc. (µg/mL)	30.50	3.98	1.99	1.50
Std. Dev. (µg/mL)	2.69	0.37	0.19	0.15
MDL (µg/mL)	7.59	1.03	0.52	0.42
LOD (µg/mL)	8.10	1.10	0.56	0.45
LOQ (µg/mL)	26.9	3.6	1.85	1.48
Conc./MDL	3.95	3.88	3.84	3.58
SNR	11.34	10.90	10.70	10.11
MDLx10 (µg/mL)	75.9	10.3	5.2	4.2
Surrogate constant (S_α)	18.23± 0.048	11.66± 0.251	1.15± 0.051	NA

NA implies parameter not applicable

Unfortunately, many developing countries do not have these institutions readily available and/or properly resourced to serve as a depot for such authentic samples. Problems with accessibility, procurement, cost of material and shipment become a burden to manufacturers, researchers and regulatory bodies who have to routinely control, monitor and regulate the quality of pharmaceuticals. Table 1 shows the unit cost of four chemical reference substances and provides the opportunity for one to figure out how much it will cost an institution in a deprived economy in a year to acquire adequate quantities of reference samples in order to assure the quality of tons of multi-source and varied pharmaceuticals that are imported or produced locally. Our study therefore explored an alternative cost effective analytical technique of equally satisfactory performance that can be used to assess the contents of pharmaceuticals without using reference chemical standards each time. Such a study is strengthened if the physico-chemical statuses of study samples are officially established as shown in Table 2.

In the development of the isocratic procedure for this work, some of the key parameters considered for satisfactory chromatography were the stationary phase, composition of the mobile phase, pH of the aqueous component of the mobile phase and the UV detection wavelength. Since HPLC is a liquid chromatographic technique, it was also necessary to find a common solvent that could dissolve study samples into a common solution. Reference to the chemical structures (Figure 1) and physico-chemical properties of the samples [13, 15, 16] helped us address the issues related to the key parameters. As a result of the reasonable polarity of the analytes, a reversed-phase chromatography was adopted. In this mode, relatively more polar analytes are eluted before the less polar because the mobile phase is usually aqueous with organic modifiers. This was what defined the retention times of the analytes in Table 2. Under the set of conditions, paracetamol was the most polar with a mean retention time of about 3.0 minutes while benzoic acid was the least polar with approximate mean retention time of 12.0 minutes. Our hypothesis was based on compounds chemically related to analyte because compounds with similar chemical structures are expected to have similar elution profiles theoretically [19] and UV absorption pattern if they are conjugated. Figure 1 showed all the compounds as conjugated and since literature [13, 15, 16] indicated the respective absorption bands of samples as potentially overlapping between 240 and 260nm, the elution was monitored at 257nm which was almost optimal for all the intersecting spectra. The summary of the chromatographic conditions therefore was a reversed-phase stationary phase, mobile phase containing methanol and 2.5%v/v glacial acetic acid (2:3), a diluent of methanol and water (1:1), flow rate of 1mL/min and UV detection wavelength as earlier stated.

The ability of an analytical procedure to accurately detect a compound present in a sample at low concentrations is a desirable property. Establishing detecting limits such as the MDL, LOD, LOQ, and SNR forms the basis for the evolving method to be fit for its intended purpose because, appropriate working concentrations of the samples can be determined after generating the adequate data for these parameters. Quality of the analytical results can therefore be assessed because the indices of analytical performance are known. Additionally, the parameters can serve as a reference for comparing results if the same method is applied by different laboratories or analysts. MDL is a statistically determined value that defines how easily measurements of a substance by a specific analytical protocol can be distinguished from measurements of a blank at 99% confidence level. In related fields such as environmental analysis where trace effluent levels are detected, it is required that the calculated

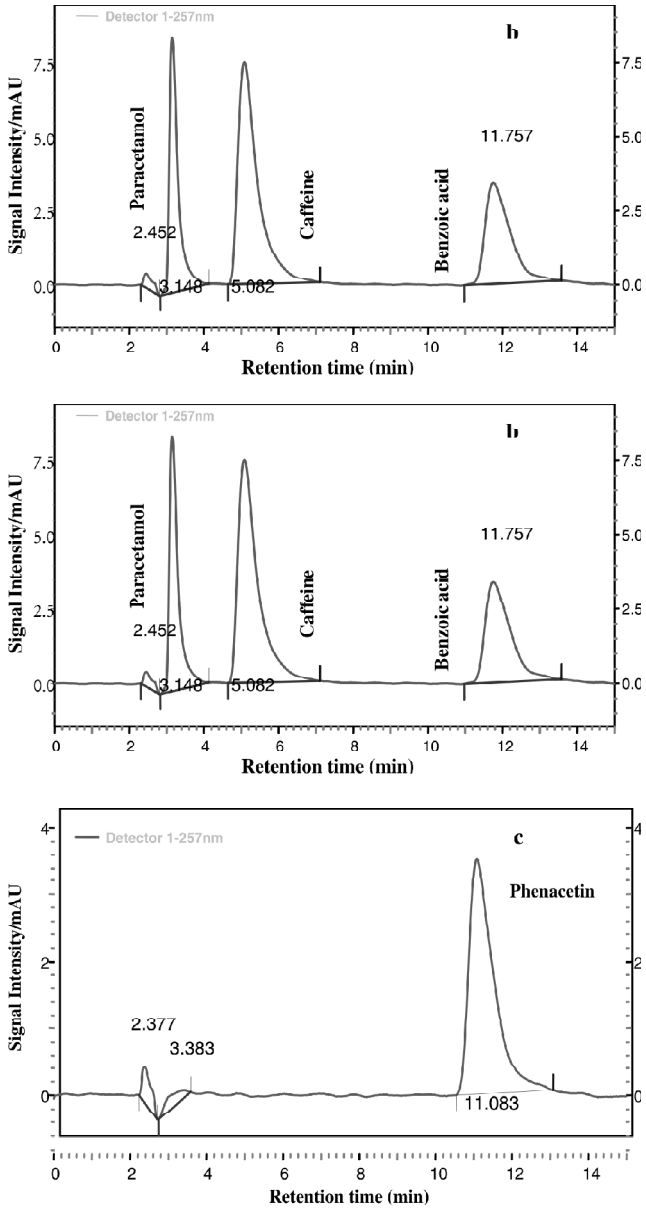

Figure 3: Representative Chromatograms of Paracetamol Caffeine and Candidate Surrogate Reference Standards. The Samples in 'a' and 'b' were Co-eluted from Respective Homogenous Stocks. 'c' was Obtained from a Solution of Phenacetin Only

MDL for an acceptable method be greater than one-tenth of the spike level [20]. This implies that the calculated MDL should be less than the spike level and ten times the calculated MDL greater than the spike level. The reason behind this analogy is that, if the MDL exceeds the spike level, it will not be possible to statistically differentiate the spiked samples from background noise.

Adopting and applying the above reference to our study, Table 4 indicates that the concentrations of samples used in determining detecting limits of the study were satisfactory as none of them was greater than ten times the calculated MDL. The importance of the above specification is that, it defines the limits within which precision of a method depends on concentration [21]. Considering the quality of signal measurement, it is often determined by the SNR and according to Skoog, Holler and Nielman [22], SNR greater than 3 means that the signal is detectable. From Table 4, SNR for all the study pure samples were between 10.0 and 12.0, suggesting that, random errors likely to be associated with a series of measurements under the proposed procedure will be low. With respect to the Conc /MDL ratio, some reports recommend a range between 1 and 5 but emphasize values within 3-5 for practical purposes [23]. The reason probably is that, a value more than 2 increases confidence in detection because the working concentration would be as far as possible from the MDL which inherently can give a false positive. The various pure samples gave values between 3.5 and 4.0 (Table 4) demonstrating that, the respective concentrations of samples were within limits of linearity of their calibration curves. The representative chromatograms in Figure 3 also provide the extent of selectivity of the analytical procedure for the samples used in the study. All the samples were reasonably resolved with no overlapping bands. The peak areas showed a proportional relationship with varying concentrations of samples above the LOD (Data not shown) making it possible for the peak areas to be used as tools for evaluating unknown concentrations of tablet samples. The internal standard however, according to Equation (1), was redundant as its effect cancelled out when the peak area ratios were factored into the equation.

The surrogate constants defined mathematically by Equation (1) for phenacetin, benzoic acid and aspirin were approximately 1, 12 and 18 (Table 4) respectively. Since this is a new study, one way of measuring the validity of surrogate constants was their respective performances according to pharmacopoeial specifications in assaying the paracetamol tablets. The requirements of the BP and USP were taken as official references. Looking carefully at Table 4, one realises that, the surrogate constants are specific and reflective of the structural differences among the candidate surrogate reference

standards. Even though benzoic acid had the lowest molecular weight (122.12g/mol), its surrogate constant was not the lowest under the study conditions. Phenacetin with molecular weight of 179.22g/mol rather had the lowest surrogate constant. Aspirin with the highest molecular weight of 180.16g/mol had the highest surrogate constant but the difference between the surrogate constants of aspirin and phenacetin compared with that of their molecular weights did not suggest molecular weight of a surrogate reference standard as a strong determinant of the magnitude of a surrogate constant. The observed trend aspirin>benzoic acid>phenacetin appeared to be a function of the ratio of the density of the particular surrogate reference to that of the principal analyte (paracetamol). Aspirin, benzoic acid, paracetamol and phenacetin respectively have the following densities: 1.350, 1.300, 1.293 and 1.240g/cm^3 (Table 2). The corresponding density ratios for aspirin, benzoic acid and phenacetin were 1.0441, 1.0054 and 0.9593, showing the same descending order as the surrogate constants. The density ratio was figured out from Equation (1) when the concentration terms were expressed as a function of density. With respect to the robustness of the surrogate constant to variations in concentration of the principal analyte, it is clear from Figure 2 that within tolerant limits, changes in concentration did not produce any linear change in surrogate constant for all the candidate surrogate reference standards. All the scatter plots were almost parallel to the horizontal axis. If signal intensity were proportional to concentration of solute within limits of linearity, then, according to Equation (1), the surrogate constant should be resilient to changes in concentration of either a principal analyte or surrogate reference candidate which is the evidence of Figure 2.

Another factor that was seen as important in relation to the magnitude of the surrogate constant was the ratio of principal analyte signal to that of candidate surrogate reference. Since UV absorption was the main mode of detection and it was assumed that signal intensity was directly proportional to concentration, the detection wavelength was a relevant condition in the estimation of this ratio. This is because, the absorptivity of various solutes at a particular wavelength may be different and this may give rise to different signal intensities and consequently, signal ratios. If the absorptivity of the principal analyte (paracetamol) at the detecting wavelength (257nm) were higher than that of the surrogate reference, the signals ratio would be high and subsequently increase the magnitude of the surrogate constant (Equation (1)) with the converse also true. These findings, therefore, suggest that, keeping all other variables constant, the absorption profile of phenacetin was closest to that of paracetamol by having a surrogate constant of about 1 while that of aspirin was most distant by having a value of about 18. This relatively huge value however, does not affect the usefulness of the surrogate reference once it is demonstrated that absorption is proportional to concentration at that wavelength. Since the findings of the study establishes that chromatographic conditions including UV detection wavelength affect the magnitude of the surrogate constant, surrogate constants must be reported in all cases with the chromatographic details.

Application of the surrogate constants to the content evaluation of the tablets produced results within the monograph requirements of the USP 2004 [24]. However, only phenacetin as a candidate surrogate reference standard provided data that was compliant with the USP and BP for all brands of tablets assayed (Table 5). Aspirin had five brands (AB, CD, IJ, MN and ST) out of nine marginally falling (106-109%w/w) without the monograph requirements of the BP (95.0-105.0%w/w) while benzoic acid had two brands (CD and ST) falling off (108 and 109%w/w). It generally appeared that, percentage contents of brands CD and ST for both the proposed and official methods were comparatively high and may have formulation elements responsible for the high assay values. As a result, the effectiveness of aspirin and benzoic acid as surrogate reference standards may not be in question with respect to brands CD and ST. Results for brand GH (Table 5) were generally lower for the proposed method (98-101%w/w) than the official methods (102-106%w/w). It was however not clear from the study if this trend had a link with the relative selectivity of the different methods. One factor that has regulatory flexibility but can potentially affect relative selectivity and accuracy of analytical methods is selection of excipients. We were not aware of the excipients used by the manufacturers and could not investigate the influence of such

Table 5
Assay of Paracetamol Tablets using Proposed and Official Methods (n=7)

	Content (%w/w)				
	Proposed Method				*Official Method*
Brand	*Aspirin*	*Benzoic acid*	*Phenacetin*	*USP*	*BP*
AB	106.14±3.432	97.55±4.540	95.63±4.016	98.18 ± 0.764	104.80±1.020
CD	106.33±6.370	108.00±1.561	97.70±3.490	98.18±0.764	104.99±0.697
EF	104.83±4.910	103.40±3.619	99.70±1.524	99.08±0.847	104.24±1.829
GH	98.85±2.996	101.90±1.987	99.25±2.761	102.32±4.364	106.67±1.598
IJ	109.55±4.174	100.40±2.339	102.00±4.844	97.53±0.587	104.24±0.607
KL	101.92±1.795	95.82±1.917	90.64±1.751	92.86±1.131	98.09±1.166
MN	106.77±3.023	98.75±1.802	94.40±2.931	108.77±1.453	102.56±1.320
QR	103.90±4.720	101.30±4.609	97.13±2.563	95.90±0.59	104.62±1.495
ST	107.43±1.174	109.30±6.931	98.60±3.758	100.93±2.805	106.48±1.154

excipients on the proposed method. However, the general assay results with the candidate surrogate reference standards did not have any brand showing a systematic deviation with respect to the pharmacopoeial specifications, it can therefore be assumed that excipients had limited or no influence on the results.

Comparing the assay results of each of the surrogate reference standards with either the BP or USP methods using the Bonferroni's multiple comparison test (Table 6), there was no significant difference at 95% confidence interval between the means of using the USP and benzoic acid or phenacetin. This signifies a positive correlation between the accuracy of using the USP method and benzoic acid or phenacetin as surrogate standards. The results of aspirin and benzoic acid were also comparable with that of the BP. However, there was a significant difference between the means of using the BP and USP, aspirin and USP and finally phenacetin and BP. In spite of the significant statistical differences in some of the assay methods, it is important to note that the proposed method produced assay results that were within monograph specifications of either the BP or USP or both. Nonetheless, the significant statistical difference realized in some cases made it difficult to establish the general relative accuracy of our proposed method to those of the pharmacopoeias. Conversely, the Bartlett's test for equal variances established the relative precision of the proposed method. There was no significant difference between the variances of all the methods at 95% confidence interval (Table 6). Our hypothesis has therefore been verified with all the candidate surrogate reference standards producing results within pharmacopoeial limits with comparable precisions. Assay results from phenacetin complied with the two official specifications, possibly emphasizing the fact that, compounds with close physico-chemical relationship to principal analyte serve as better surrogate reference standards. Of all the candidate surrogate reference compounds, phenacetin has more to share both physico-chemically and pharmacologically with paracetamol.

Finally, the study provided the following general guidelines for the application of the proposed method in pharmaceutical analysis:

- The candidate surrogate reference standard and principal analyte must be physico-chemically equivalent.
- Signals of surrogate reference standard and principal analyte should be linearly related to the physical property giving rise to instrumental responses.
- Internal standards are not required as both the surrogate reference standard and principal analyte compensate for each other.
- Method is limited to only conditions where the surrogate reference standard and principal analyte can be resolved simultaneously.
- Surrogate constants should be established with previously available chemical reference standards and should always be reported with the chromatographic details.

Table 6
Statistical Analysis of Means and Variances of Proposed and Official Methods

Bonferroni's Multiple Comparison Test					
Test	*Mean Diff.*	*t*	*Significant? P < 0.05?*	*Summary*	*Bartlett's test for equal variances*
Aspirin vs USP	5.774	4.680	Yes	***	
Aspirin vs BP	1.226	0.8109	No	ns	
Benzoic Acid vs USP	2.519	2.041	No	ns	
Benzoic Acid vs BP	-2.030	1.343	No	ns	
Phenacetin vs USP	-2.078	1.684	No	ns	
Phenacetin vs BP	-6.627	4.385	Yes	***	
USP vs BP	-4.549	3.010	Yes	*	
Bartlett's statistic (corrected)					4.933
P value					0.2942
P value summary					ns
Variances differ signif.					No

- Preferrably, surrogate reference substances should have a sufficiently high state of purity.

Further work is looking at a broader range of formulations and surrogate candidates to validate some of the earlier findings and define the rule of thumb for the candidate method.

CONCLUSION

Surrogate reference standards have successfully been applied to the assay of paracetamol tablets. It has shown the potential for use in routine quantitative HPLC applications once the HPLC method is evolved and the surrogate constant ($S_{á}$) is determined with previously available chemical reference standard.

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