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A New Dimension in Medicines Quality Monitoring: Using Surrogate Reference Standards in RP-HPLC Assay of Pharmaceuticals

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ABSTRACT: To improve the efficiency of medicines quality monitoring in developing countries by reducing the limitations of lack of regular access to chemical reference standards(CRS) and corresponding financial burden, a new approach of using readily available compounds, physico-chemically related to the active pharmaceutical ingredient (API) as surrogate reference standards in reverse-phase HPLC (RP-HPLC) assays has been reported. Isocratic RP-HPLC methods were designed and validated for 7 APIs, using their respective reference standards and compounds designated as candidate surrogate reference standards to determine 'surrogate constants' (S_α) for each of the surrogate reference standards with respect to a particular API. 35 pharmaceutical products containing the different APIs from 23 manufacturing outfits (foreign & local) were assayed with the surrogate reference standards, putting the S_α into a previously reported equation to determine the percentage content of the test samples. Assay results from the proposed method were compared statistically with standard methods of the British Pharmacopoeia (BP 2007) and the International Pharmacopoeia (IP 2011) where appropriate. All the pharmaceutical products were successfully assayed with surrogate reference standards and the assay results were statistically comparable with those of the BP 2007 and IP 2011. Eleven compounds (11) of either analytical or pharmaceutical grade were used as surrogate reference standards for the 7 APIs with each API having more than one compound as surrogate reference standard. Some compounds could serve as surrogate reference standards (ascorbic acid, benzoic acid, paracetamol, piroxicam and metronidazole) for other APIs as well.

Keywords: Surrogate reference standard, surrogate constant, pharmaceuticals, pharmaceutical grade, chemical reference standard.

INTRODUCTION

The *'silent murder'* caused by counterfeit, adulterated and sub-standard medicines continues to be a problem in Sub-Saharan Africa and other developing nations. In countries where there is liberalization of trade and import laws such as Ghana, there is influx of pharmaceuticals on to the local market with attendant problems with quality. The high humidity and temperatures prevalent in Sub-Saharan Africa and other developing countries also affect the stability and proper storage of some pharmaceutical products, contributing to the menace of poor quality medicines. However, a key step generally in monitoring the quality of medicines is acquisition of sufficient and reliable assay data among others, to check for compliance of medicines with pharmacopoeial monograph specifications. The RP-HPLC technique has been a useful tool in the acquisition of both qualitative and quantitative data for *in vitro* as well as *in vivo* quality assessments [1, 2, 3 & 4] and a lot of current assay methods in the pharmacopoeias (BP &USP) are based on RP-HPLC.

However, the application of RP-HPLC in the analyses of medicines usually requires the use of chemical reference standards (CRS) of the active pharmaceutical ingredient (API) to estimate the percentage content of the manufacturer's claim. Useful as CRS have been in the monitoring of quality of medicines globally, easy accessibility and cost (product & import) of CRS have been a great challenge to medicines quality institutions in developing nations who have to regularly monitor and control the quality of multi-source plethora of medicines on their national markets as a result of liberal trade and import laws.

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Consequently, the regulation of the quality of pharmaceuticals is further weakened with increased *'silent murder'* due to circulation of poor quality, sub-standard medicines. Table 1 is an overview of the cost involved in procuring reference standards for the regular quality monitoring of the APIs in pharmaceutical products used in this study. It is realized from the Table that, CRS for only 8 different pharmaceutical products may cost medicines regulatory/quality research institutions about $2000.00 for only a unit each of the listed items without cost of courier services and import taxes. When the diversity of pharmaceutical products on the market of a developing country and the volume of work involved in conducting both pre-and post-market surveillance are considered in the light of limited budgetary allocation from governments to medicines regulatory/quality research institutions, the limitations to regular and effective quality monitoring of pharmaceuticals become deepened.

In an attempt to overcome the limitation of lack of regular access to CRS in drug analyses and to strengthen cost effective management of the quality of pharmaceuticals in Sub-Saharan Africa, we had in a preliminary study [5] examined the use of compounds chemically related to target analytes as surrogate reference standards in quantitative HPLC. In this context, a suitable surrogate reference standard should be physico-chemically similar to the analyte of interest. The RP-HPLC procedure should simultaneously elute, identify and quantify both the target analyte and surrogate reference standard (pure compound of either analytical or pharmaceutical grade) without interference from either of them. By physico-chemical similarity, we had reported that a surrogate reference standard should have similar solubility and detection properties as analyte of interest with a demonstration of linear response between concentration and a measurable physical property of the analyte of interest [5].

The following theory, which was established and also reported in our preliminary study, underpins how (S_α) can be used to determine the percentage content of pharmaceuticals:

$$A_t / C_t = S_\alpha (A_s/C_s) \quad (1)$$

S_α is a dimensionless constant of proportionality that we have previously reported as the surrogate constant; A_t and A_s are the respective signal intensities of a target analyte and a surrogate reference standard with C_t and C_s as corresponding concentrations. C_t which can also be referred to as the actual concentration of the target analyte when a pharmaceutical formulation is being analysed can be determined, when the other variables in Equation 1 are known. Nominal concentrations are usually 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 of the medicinal sample being analysed. We have already reported the assay of brands of paracetamol tablets with surrogate reference standards [5].

The current study examined a wider scope of pharmaceuticals with different active ingredients, dosage forms (tablets, capsules, oral solutions and infusions), strengths (4-500mg) and manufacturers. The potential effect of each of these variables on the robustness and effectiveness of our procedure was of much interest and keenly monitored. The relative precisions and accuracies of our assays with respect to those of the pharmacopoeias (BP & IP) were also tested. Since we are still defining the rule-of-thumb for the best surrogate reference standard, a minimum of two

Table 1
Price Quotes for Some Chemical Reference Standards from the USP Daily Reference Standards Catalog compiled between 2010 and 2012

Catalogue No.	*Reference Standard*	*Current Lot*	*Quantity / unit*	*Unit Price ($)*
1123000	Chlorpheniramine Maleate	N0G316	125mg	158.00
1134335	Ciprofloxacin HCl	JOH307	400mg	204.00
1185008	Diazepam	I2G270	100 mg	263.00
1341001	Indometacin	J1G345	200 mg	199.00
1356836	Lamivudine	H01378	200mg	204.00
1396309	Metformin HCl	I0H236	200mg	233.00
1442009	Metronidazole	J11272	100mg	199.00
1544508	Piroxicam	H2H258	200mg	199.00

compounds was used as candidates for each group of API analysed.

EXPERIMENTALS

Materials/ Reagents

The reagents and solvents listed below were provided by the Department of Pharmaceutical Chemistry, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana: Methanol (BDH), Glacial acetic acid (BDH), Acetic anhydride (BDH), Benzoic acid (BDH), Sodium hydroxide (BDH), Hydrochloric acid (BDH), Ethanol (BDH), Sulphuric acid (BDH), Perchloric acid (Sigma-Aldrich), Phenolphthalein indicator (In-house), Acetone (Fisher), Potassium dihydrogen orthophosphate anhydrous (Merck), Phenol red indicator (In-house), Sulphamic acid (Sigma-Aldrich), Anhydrous Formic Acid (BDH), Acetonitrile (BDH), n-Butanol (Sigma-Aldrich), Sodium Acetate anhydrous (Fisher), Toluene (BDH), Iodine crystals (Sigma-Aldrich), Starch solution (In-house), Dragendorff's reagent (In-house), Cerium (IV) Sulphate (BDH), Diethyl ether (Fisher), Ethyl acetate (Fisher), Ammonium hydroxide (Fisher), Potassium hydrogen phthalate (BDH), Sodium thiosulphate (BDH), Potassium dichromate (BDH), Crystal-violet indicator (In-house), Ammonia (BDH) and Mercuric acetate (Fisher).

The solvents were mostly of research grade except the HPLC grade for HPLC analyses. The reagents were of analytical grade with in-house distilled/deionised water. Details about the pure reference powders (target analytes and surrogate reference candidates) and pharmaceutical products have been presented in Tables 2&3. The pharmaceutical products were bought from registered retail Pharmacies in Kumasi, 2nd largest city of Ghana, between 2009 and 2012 with each product having one year or more of its shelf-life remaining as of the time of purchase. Some of the pure reference powders were provided by the Food and Drugs Authority, Ghana, and some Pharmaceutical Industries in Ghana. All pure reference powders and pharmaceutical products were used before expiry.

Instrumentation

The liquid chromatograph consisted of Shimadzu LC-10AS Liquid Chromatograph Pump, ODS columns (See Table 4), Applied Biosystems 783A Programmable Absorbance Detector and HP Computer Work Station with eDAQ Power Chrom Software 280 for chromatograms and integration. Other equipment includes Griffin Flask Shaker, EUTECH Instruments Cyberscan pH meter,

Table 2
Profile of Pure Reference Powders (Target and Surrogate Analytes) used in the Study

Sample	*Batch No.*	*Expiry Date*	*Melting Range (°C)[a]*	*Mean % Purity*
Ascorbic acid	0011847	06/2012[b]	190-191 (ca 190)	99.27 (99.0-100.5)
Benzoic acid	09K207004	02/2014	122-124 (121-124)	99.65 (99-100.5)
Caffeine anhydrous	0912007-P1019	12/2013	236-239 (234-239)	98.71 (98.5-101.5)
Chlorpheniramine Maleate	BL/SC/C/0608012	04/2013	132-135 (130-135)	98.34 (98.0-101.0)
Ciprofloxacin HCl	101119-2	11/2013	254-256 (255-257)	99.40 (99.0-101.0)
Diazepam	20080204	02/2011[b]	132-134 (131-135)	100.10 (99.0-101.0)
Indometacin	X061210	11/2010[b]	158-160 (158-162)	99.10 (98.5-100.5)
Lamivudine	LU1661011	09/2013	178-180 (174-180)	99.86 (97.0-103-0) (IP 2011)
Metformin HCl	Q137	02/2012[b]	223-225 (222-226)	99.50 (98.5-101.0)
Metronidazole	09011801	01/ 2013	160-162 (159-163)	99.90 (99.0-101.0)
Naproxen	0903201	03/2012[b]	154-156 (154-158)	99.80 (99.0-101.0)
Paracetamol	J8-299	12/2011[b]	168-170 (168-172)	99.54 (99.0-101.0)
Piroxicam	B/NK8-19	03/2012[b]	192-194 (200-202)	100.08 (98.5-101.0)
Salicylic acid	300384B	-	157-158 (158-161)	99.00(99.0-100.5)

a: Data in parenthesis are pharmacopoeial reference values, mostly the British Pharmacopoeia 2007

b: Work was completed before the expiry of samples

Table 3
Profile of Pharmaceutical Products Studied

Product[c]	*Batch No.*	*Expiry Date*	*Dosage Form*	*Strength*
Chlorpheniramine Maleate, BP (PGL)	B9006	06/2013	Tablet	4mg
Chlorpheniramine Maleate, BP (KPL)	10119	09/2013	Tablet	4mg
Chlorpheniramine Maleate, BP(LPL)	110084	12/2012	Tablet	4mg
Chlorpheniramine Maleate, BP (EPL)	50.001	11/2012	Tablet	4mg
Ciprofloxacin (UCL)	11	04/2014	Tablet	500mg
Ciprofloxacin (MVL)	PC2111001A	03/2014	Tablet	500mg
Ciprofloxacin (DPL)	12050905	05/2015	Tablet	500mg
Ciprofloxacin (SLL)	CP-01	03/2014	Tablet	500mg
Ciprofloxacin (ECL)	1011L	11/2015	Tablet	500mg
Ciprofloxacin (ECL	C-10/11/101	05/2014	Infusion	2mg/mL
Ciprofloxacin (MBL)	22110648	11/2014	Infusion	2mg/mL
Ciprofloxacin (SPL)	82EK423009	09/2014	Infusion	2mg/mL
Ciprofloxacin (LPU)	82EF423001	05/2014	Infusion	2mg/mL
Diazepam (GTL)	DZ5015	11/2011	Tablet	5mg
Diazepam (PGL)	8008	03/2012	Tablet	5mg
Diazepam (ECL)	0110H	10/2012	Tablet	10mg
Indometacin (LPL)	019046	11/2011	Capsule	25mg
Indometacin (ECL)	**	**	Capsule	25mg
Indometacin (MGP)	**	**	Capsule	25mg
Lamivir	KT9345	09/2012	Tablet	150mg
Zeffix	R504490	11/2013	Tablet	100mg
Lamdek	1206157	06/2014	Tablet	150mg
Lamivudine (CLI)	G00136	07/2011	Oral Soln.	10mg/mL
Metformin HCl, BP ((HVD)	BA05407	05/2013	Tablet	500mg
Metformin HCl, BP (DNK)	680	01/2015	Tablet	500mg
Metformin HCl, BP (PDR)	091001	10/2012	Tablet	500mg
Metformin HCl, BP (ECL)	4112K	12/2014	Tablet	500mg
Metronidazole (ECL)	0701L	06/2015	Tablet	200mg
Metronidazole (LPL)	0740881	03/2013	Tablet	200mg
Metronidazole (MGP)	MZ119	02/2014	Tablet	200mg
Metronidazole (MLI)	XT015	05/2013	Tablet	200mg
Piroxicam (ECL)	0508J	08/2013	Capsule	20mg
Piroxicam (KPL)	10012	11/2013	Capsule	20mg
Piroxicam (LPL)	0210131	02/2013	Capsule	20mg
Piroxicam (LPU)	100428	03/2013	Capsule	20mg

c: Generic products were differentiated by adding three letters from the name of the manufacturing company in parenthesis
**Information was not available on product

Stuart Melting Point SMP 10 Apparatus, T_{90} + UV/VIS Spectrometer; PG Instruments Limited, Buchi Rotary Evaporator, Adam PW/24 Analytical weighing balance, Chromato-Vue C-70 UV View System (UVP Inc) and Clifton Sonicator, Nickel – Electro Limited.

Methods

The pure reference powders (target analytes), surrogate reference standards and pharmaceutical products were individually characterized according to the requirements of their respective monographs in the BP 2007 [6] or IP 2011 [7]. These included colour reactions, melting point determinations, thin layer chromatography assessments, assays for purity (pure reference powders) and uniformity of weight tests (Tablet dosage forms). For the proposed method, a number of isocratic HPLC analytical methods were developed and validated for the various groups of APIs and corresponding surrogate reference standards. Summarized details of the HPLC

Table 4
Structural Formulae and Some Relevant Physico-chemical Properties of Compounds used (APIs and Surrogate Reference Standards)

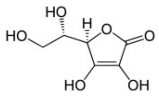
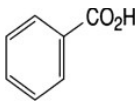
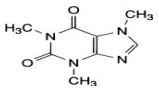
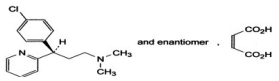
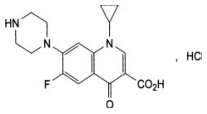
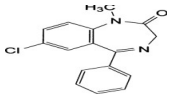
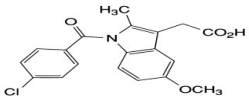
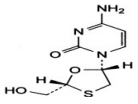
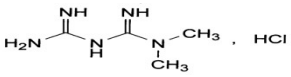
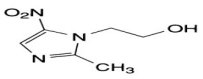
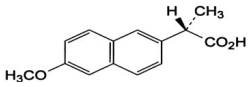
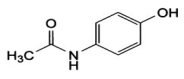
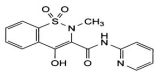
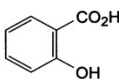
Compound	*Structural Formula*	*Molecular weight (g/mol)*	*pka*	*Solubility in water (g/L) (20-25°C)*
Ascorbic acid		176.1	4.17 and 11.57 [10]	333.33 [9]
Benzoic acid		122.1	4.20 [11]	2.90 [12]
Caffeine		194.2	14.0 [13]	16 [13, 14]
Chlorpheniramine Maleate		274.8	9.20 and 4.00 [15]	10-50 [16]
Ciprofloxacin HCl		331.3	6.80 and 8.73-8.76 [17,18]	10-30 [19]
Diazepam		284.7	3.40 [20]	0.05 [21]
Indometacin		357.8	4.50 [22]	0.0009 [23]
Lamivudine		229.3	4.30 [24]	70 [24]
Metformin HCl		129.2	12.40 [25]	>300.[26]
Metronidazole		171.1	2.62 [27]	10.5 [27]
Naproxen		230.3	4.15 [28]	0.016 [28]
Para aminophenol		109.1	5.48, 10.46 [29]	15 [29]
Paracetamol		151.2	9.5 [31]	14.90 [30]
Piroxicam		331.3	5.46, 1.86 [32]	0.19 [33]
Salicylic acid		138.1	2.98 [11]	1.60[34]

Table 5
Details of Designed HPLC Methods, Retention Times, Surrogate Constants and Assay Results using Surrogate Reference Standards and Pharmacopoeial Methods

Chromatographic Details	Target & Surrogate samples	Analyte conc. (µg/mL)[e]	Mean retention times±sd (min)(n=5)	Regression data (r^2) Concentration: %w/v	Mean surrogate constants (S_0)±sd (n=5)	Mean % content±sd ($6 \leq n \leq 10$)	
						Candidate Methods	BP 2007/IP 2011[f]
ODS C-18 Phenomenex 250x4.6mm column; Methanol /0.025M Phosphate buffer (pH 6.4)(1:1 v/v); 1mL/min; 266nm	Chlorpheniramine Maleate[d]	52.5	2.6 ± 0.09	y=879.3x-0.965 (0.998)	(-)	(-)	99.5±3.60
	Ascorbic acid (ASC)	11.0	3.2 ± 0.02	y=5526x-0.366 (0.999)	0.1560 ± 0.002	98.4±3.63	(92.5-107.5)
	Caffeine (CAF)	32.0	5.9 ± 0.02	y=62043x+1.217 (0.997)	0.2224 ± 0.006	99.3±3.58	
	Piroxicam (PIR)	70.0	6.5 ± 0.07	y=554.9x+0.898 (0.996)	0.8095 ± 0.003	99.5±4.25	
ODS C-18 Phenomenex 250x4.6mm column; Methanol/Water/0.025M Phosphate buffer (pH3.0)(10:9:1 v/v/v); 1.5mL/min; 239nm	Ciprofloxacin HCl (Tab.)[d]	50.0	2.7±0.20	y=97.5x-0.425 (0.997)	(-)	(-)	97.9±1.81
	Benzoic acid (BEN)	50.0	4.7±0.20	y=214.5x+0.451 (0.996)	0.3921±0.008	97.9±1.48	(95.0-105.0)
	Salicylic acid (SAL)	50.0	4.4±0.15	y=159.5x+0.509 (0.995)	0.4602±0.010	97.5±1.57	
	Ciprofloxacin Lactate (IV)[d]	50.0	2.7±0.20		(-)	(-)	97.9±1.42
	Benzoic acid (BEN)	50.0	4.7±0.20			97.7±1.42	(95.0-105.0)
	Salicylic acid (SAL)	50.0	4.4±0.15			97.1±1.87	
Hichrom ODS C-18 250x4.6mm column; Methanol/0.05M Phosphate buffer (pH 5.8)(3:1 v/v); 1mL/min; 300nm	Diazepam[d]	8.0	6.7±0.21	y=96726x-6.063 (0.998)	(-)	(-)	97.2±2.01
	Indometacin (IND)	4.0	2.9±0.11	y=413455x-8.438 (0.996)	0.3230±0.018	95.3±1.82	(92.5-107.5)
	Metronidazole (METR)	4.0	2.2±0.15	y=892353x-6.754 (0.998)	0.1353±0.009	99.0±3.76	
	Piroxicam (PIR)	1.6	2.7±0.07	y=640199x-11.962 (0.993)	0.2042±0.002	97.9±3.63	
Hichrom ODS C-18 250x4.6mm column; Methanol/0.05M Phosphate buffer (pH 5.8)(3:2 v/v); 1.5mL/min; 254nm	Indometacin[d]	3.7	5.4±0.12	y=254524x-2.817 (0.998)	(-)	(-)	96.6±2.90
	Benzoic acid (BEN)	9.0	1.7±0.05	y=113304x-8.896 (0.999)	3.4260±0.073	97.5±1.84	(90.0-110.0)
	Naproxen (NAPR)	3.2	2.6±0.03	y=212757x-11.856 (0.997)	1.6735±0.023	97.1±1.68	
Luna 5µ C18 (2), 150 x 4.6 mm column; 1%v/v Acetic acid/Methanol (17:3 v/v); 1mL/min; 280nm	Lamivudine (Tab.)[d]	3.0	1.9±0.06	y=41453x-0.023 (0.997)	(-)	(-)	99.5±1.69
	Metronidazole (METR)	10.0	5.0±0.33	y=12318x+0.802 (0.998)	3.2398±0.025	98.8±1.82	(90.0-110.0) IP
	Paracetamol (PAR)	15.0	5.2±0.11	y=144.9x+0.875 (0.999)	4.6660±0.012	99.7±2.15	99.7±0.05
	Lamivudine (Oral solution)[d]	3.0	1.9±0.06		(-)	(-)	(90.0-110.0) IP
	Metronidazole (METR)	10.0	5.0±0.33			99.6±0.17	
	Paracetamol (PAR)	15.0	5.2±0.11			99.5±0.09	
ODS C-18 Phenomenex 250x4.6mm column; Methanol/0.025M Acetate buffer (pH 5.1)(3:7 v/v); 1mL/min; 245nm	Metformin HCl[d]	57.6	3.4 ± 0.03	y=175.3x-7.014 (0.997)	(-)	(-)	100.1±2.83
	Paracetamol (PAR)	57.6	4.6 ±0.02	y=113.3x+2.481 (0.996)	0.8623 ± 0.020	100.8±1.95	(95.0-105.0)
	Metronidazole (METR)	57.6	5.3 ± 0.20	y=207.9-9.658 (0.995)	1.3262 ± 0.020	101.1±3.58	
ODS C-18 Phenomenex 250x4.6mm column; Methanol/Water (3:7 v/v); 1.5mL/min; 254nm	Metronidazole[d]	50.0	4.2±0.02	y=1004x+0.243 (0.999)	(-)	(-)	101.1±3.33
	Ascorbic acid (ASC)	50.0	1.8±0.01	y=1894x-1.25 (0.995)	0.6763±0.004	101.0±3.11	(95.0-105.0)
	Para aminophenol (PAP)	50.0	2.8±0.02	y=809x+0.118 (0.998)	1.1229±0.016	101.1±3.19	
ODS C-18 Phenomenex 250x4.6mm column; Methanol/0.05M Phosphate buffer (pH 6.2) (1:1 v/v); 1mL/min; 254nm	Piroxicam[d]	20.0	6.8±0.07	y=3988x+0.367 ((0.998)	(-)	(-)	98.9±1.87
	Ascorbic acid (ASC)	20.0	2.6±0.06	y=12558x+1.372 (0.998)	0.4569±0.006	98.8±1.76	(95.0-105.0)
	Metformin HCl (METF)	20.0	3.4±0.05	y=8956x-0.318 (0.999)	1.6200±00.006	97.9±2.69	
	Metronidazole (METR)	20.0	4.1±0.06	y=5327x+0.515 (0.999)	1.9411±0.004	98.7±2.09	

[d]Target analyte
[e]Equal volumes of Target analyte and Surrogate reference standard were taken
[f]Pharmacopoeial reference values in parenthesis; (-) Data not applicable

analytical procedures have been presented in Table 5. Official pharmacopoeial methods of identification and assays [6&7] were also applied to all the pharmaceutical products without modification and the correlation between assay results (accuracy and precision) of the candidate and official methods statistically evaluated.

STATISTICAL ANALYSES

Graph Pad Prism Version 5 was used for both descriptive statistics (means, standard deviations, outliers) and test of hypotheses (Bonferroni's multiple comparison tests and One-Way Analyses of Variance).

RESULTS AND DISCUSSION

Development and Optimization of Cost Effective RP-HPLC Methods

One of the key elements of effective pharmaceutical supplies in Primary Health Care is the provision of appropriate quality medicines that are safe and efficacious to patients. Therefore, procedures and methodologies for assessing and monitoring medicines quality should be as simple, accessible and inexpensive as possible to make quality control of medicines highly operational in developing countries. In addition to mitigating by our proposed concept the constraints of lack of or limited access to CRS on effective regular quality assessment exercises in developing countries, we also designed and validated (details of validation not presented in this paper) for our assays RP-HPLC procedures of comparable pharmacopoeial accuracies and precisions (Tables 5&6) with reagents and solvents that can be readily available in reasonable levels of purity in developing countries (Table 5). It was realized that different combinations of methanol, distilled/deoinised water, phosphate/acetate buffers and acetic acid provided suitable conditions of separation and quantification with mean retention times between 1.7-6.8 min on mostly 25.0cm ODS columns for all the APIs and surrogate reference standards (Table 5) without necessarily using very expensive reagents and complex technologies as applicable in some of the pharmacopoeial methods of assay. A maximum HPLC run time of about 7.0min (Table 5) also appears very good for repeated measurements and large sample sizes on routine basis.

Quality Assurance of the RP-HPLC Method Development Process

As a quality assurance measure, all the chemical samples (APIs and surrogate reference standards) and pharmaceutical products were officially characterized according to their respective pharmacopoeial monograph requirements and found compliant before being included in the study. All colour reactions and TLC profiles of samples were correctly confirmed as specified in monographs (Data not shown). Details of melting range and purities of samples are as indicated in Table 2 and it is evident that all the samples (target analytes and surrogate reference standards) were within limits of their respective monograph specifications to rule out potential errors in the method development due to defective identity and purity.

UV Detection and Demonstration of Linearity between Analyte Concentrations and UV Absorbance

Considering a measurable physical property of the target analyte and surrogate reference samples, all the compounds had reasonable chromophores (Table 4) [6, 7&8] that made UV detection possible. Since the relationship between concentration and signal intensity is critical according to Equation 1 [5] in determining the surrogate constant (S_α), which is also very critical in the percentage content determination of samples, the UV detection wavelength becomes an important parameter in the application of our concept. The wavelength of detection for a set of target analyte and its surrogate reference compound(s) should be such as allow for quantitative determination of both compounds at varying concentrations. It is therefore necessary for the selected compounds to demonstrate linearity within the linear dynamic region of the UV detector at the selected wavelength. This, in addition, helps in choosing the appropriate working concentration(s) for both the target analyte and the surrogate reference standard.

In Table 5, details of the regression data including coefficient of correlation (r^2) for all the chemical samples studied indicate linear relationships between concentration and detector response ($0.995 \leq r^2 \leq 0.999$). Within the linear dynamic region of the detector, different concentrations of target analyte and surrogate reference standard under the same set of

chromatographic conditions have been demonstrated to give approximately the same S_{α}. (Table 5). This is possible because from Equation 1, S_{α} is determined by the product of ratios of concentration and detector responses of constituent analytes of the solution.

If detector response varies linearly with concentration, then the ratios for each constituent analyte within the equation will be the same irrespective of the analyte concentration. It therefore makes the procedure kind of rugged to variations in concentrations of constituent analytes once they are within the region of detector linearity.

The Theory and Application of the Surrogate Constant

With respect to the surrogate constants, a particular target analyte can have more than one surrogate reference standard with different surrogate constants in relation to the different surrogate reference standards, whether the chromatographic conditions are the same or different (Table 5). We had earlier reported three surrogate reference standards with corresponding surrogate constants for paracetamol using the same chromatographic conditions [5]. It is important to note that, different dosage forms of the same API does not change the surrogate constant for identified surrogate reference standards once the HPLC method is selective and specific for the API and the chromatographic conditions are maintained. We have demonstrated this with the assay of ciprofloxacin hydrochloride tablets and infusions as well as lamivudine tablets and oral solution (Table 5). However, different electronic, mesomeric and acid-base behaviours of substances (Table 4) in solution make different surrogate standards have different surrogate constants under the same or different chromatographic conditions for the same target analyte (Table 5) under UV detection. This explains why in Table 5, the different surrogate reference standards for the same API have different surrogate constants even though the same chromatographic conditions were applied. Notwithstanding that, the surrogate constant of a surrogate reference standard with respect to a target analyte is practically constant once the chromatographic conditions that were used to determine the constant are maintained. In that regard, once the surrogate constant has been determined for a surrogate reference standard with a previously available CRS of the API, subsequent assays of pharmaceutical products containing the API can be carried out without the CRS but the surrogate reference standard and the surrogate constant with the same chromatographic conditions that established the constant.

Assay of Pharmaceutical Products

The content of the active ingredient in a pharmaceutical product is a critical index in successful drug therapy. This is because a sub-therapeutic, therapeutic or toxic dose of a pharmaceutical agent is a function of the plasma concentration of the active ingredient. Sub-therapeutic doses of antibiotics can lead to treatment failures with possible development of microbial resistance while a slight over-dose of agents with narrow therapeutic window such as digoxin or amphotericin B can be toxic to patients. It is therefore desirable of an analytical technique for content evaluation of pharmaceuticals to have a wide scope of application in terms of chemical compounds, dosage forms and strength of unit dose among others. Our attempt at investigating the versatility of our concept is illustrated by Table 3 which profiles the range of pharmaceutical products used in this study. Samples of analgesics, antibiotics, sedative-hypnotics & muscle relaxants, anti-diabetics and anti-retroviral have been successfully assayed with results that are statistically comparable with those of the pharmacopoeias (Tables 5&6). Each of the substances considered for a surrogate reference compound produced assay results that statistically correlated with the corresponding pharmacopoeial method both in terms of precision and accuracy (Table 6). The Bonferroni's Multiple Comparison Test (Table 6) also revealed that among the various compounds used as surrogate reference standards for a particular API, assay results were statistically comparable. This therefore demonstrates the potential cost-effectiveness, convenience and affordability of our technique. Meanwhile, the study continues with additional pharmaceutical agents and formulations to identify further strengths and potential limitations.

Table 6
Relative Precision and Accuracy of the Proposed Method and Pharmacopoeial Methods

Bonferroni's Multiple Comparison Test (95% Confidence Interval)						Analysis of Variance
Product	**Paired Tests**	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**	**Significant? P < 0.05? ns? P > 0.05?**
Chlorpheniramine Maleate Tabs.	BP vs. ASC	1.1200	0.3632	No	ns	
	BP vs. CAF	0.1850	0.0600	No	ns	P value: 0.9790
	BP vs. PIR	-0.0250	0.0081	No	ns	P value summary: ns
	ASC vs. CAF	-0.9350	0.3032	No	ns	F: 0.0617
	ASC vs. PIR	-1.1450	0.3713	No	ns	
	CAF vs. PIR	-0.2100	0.0681	No	ns	
Ciprofloxacin HCl Tabs.	BP vs. BEN	-0.0120	0.0104	No	ns	P value: 0.9101
	BP vs. SAL	0.4280	0.3722	No	ns	P value summary: ns
	BEN vs. SAL	0.4400	0.3826	No	ns	F:0950
Ciprofloxacin Infusion	BP vs. BEN	0.1150	0.0863	No	ns	P value: 0.8172
	BP vs. SAL	0.7925	0.5946	No	ns	P value summary: ns
	BEN vs. SAL	0.6775	0.5083	No	ns	F: 0.2065
Diazepam Tabs.	BP vs. IND	1.9070	0.6473	No	ns	P value: 0.6563
	BP vs. METR	-1.7930	0.6088	No	ns	P value summary: ns
	BP vs. PIR	-0.7267	0.2467	No	ns	F: 0.5598
	IND vs. METR	-3.7000	1.2560	No	ns	
	IND vs. PIR	-2.6330	0.8940	No	ns	
	METR vs. PIR	1.0670	0.3621	No	ns	
Indometacin Caps.	BP vs. BEN	-0.9100	0.3814	No	ns	P value: 0.9841
	BP vs. NAPR	-0.5233	0.2193	No	ns	P value summary: ns
	BEN vs. NAPR	0.3867	0.1621	No	ns	F: 0.0501
Lamivudine Tabs.	IP vs. METR	0.6800	0.3584	No	ns	P value: 0.9003
	IP vs. PAR	0.1400	0.0738	No	ns	P value summary: ns
	METR vs. PAR	-0.8200	0.4322	No	ns	F: 0.1069
Lamivudine Oral Solution	IP vs. METR	0.1360	0.8352	No	ns	P value: 0.4830
	IP vs. PAR	0.1980	1.2160	No	ns	P value summary: ns
	METR vs. PAR	0.0620	0.3808	No	ns	F: 0.7738
Metformin HCl Tabs.	BP vs. METR	-1.0050	0.4267	No	ns	P value: 0.9073
	BP vs. PAR	-0.7500	0.3184	No	ns	P value summary: ns
	METR vs. PAR	0.2550	0.1083	No	ns	F: 0.0984
Metronidazole Tabs.	BP vs. ASC	0.0825	0.0313	No	ns	P value: 0.9993
	BP vs. PAP	-0.0075	0.0029	No	ns	P value summary: ns
	ASC vs. PAP	-0.0900	0.0342	No	ns	F: 0.0007
Piroxicam Caps.	BP vs. ASC	0.1400	0.0804	No	ns	P value: 0.9413
	BP vs. METR	0.2400	0.1378	No	ns	P value summary: ns
	BP vs. METF	0.9875	0.5672	No	ns	F: 0.1286
	ASC vs. METR	0.1000	0.0574	No	ns	
	ASC vs. METF	0.8475	0.4868	No	ns	
	METR vs METF	0.7475	0.4294	No	ns	

CONCLUSION

Surrogate reference standards in HPLC application have a great potential in pharmaceutical analyses especially in resource-constrained countries where sub-standard and counterfeit medicines continue to be a threat. We have successfully tested our hypothesis with 11 surrogate reference standards against 7 APIs (chlorpheniramine maleate, ciprofloxacin hydrochloride, diazepam, indometacin, lamivudine, metformin, metronidazole and piroxicam), 35 pharmaceutical products containing the various APIs, 4 dosage forms (capsules, infusions, oral solution and tablets), unit dose strength between 4-500mg and medicinal products from 23 Manufacturers (both foreign and local). Each of the APIs had more than one surrogate reference standard with some of the surrogate reference standards being suitable for

other APIs under different chromatographic conditions. Institutions responsible for the quality of medicines in developing countries can therefore identify and establish surrogate reference standards suitable for their scope of work and validate the surrogate constants in other similar laboratories for their internal use. This provides a viable alternative to the high cost of and lack of regular access to CRS for efficient pre-registration and post-market surveillance of the quality of medicines.

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Conflict of Interest

The authors of this study declare no conflict of interest

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