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Reliable low-cost capillary electrophoresis device for drug quality control and counterfeit medicines

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ABSTRACT

The proportion of counterfeit medicines is dramatically increasing these last few years. According to numerous official sources, in some pharmaceutical wholesalers in African countries, the proportion has reached 80%. Unfortunately, this situation is far to be improved due to lack of suitable analytical equipment allowing rapid actions of the Regulatory Agencies based on scientific consideration, at affordable cost and all over the drug supply chain.

For that purpose, a network group considered that mater by building a low-cost original capillary electrophoresis (CE) equipment equipped with a new deep UV detector based on LED technology.

The generic conditions for analysis were investigated: capillary zone electrophoresis (CZE) performed at acidic pH for basic drug molecules (i.e., quinine, highly used as the last antimalarial rampart), basic pH for compounds such as furosemide (a common diuretic drug) and at neutral pH for a well known antibiotic combination, trimethoprim/sulfamethoxazol.

To evaluate the ability of the CE equipment for quantification, a full validation and a method comparison study were carried out for the CZE method dedicated to quinine determination. The validation involved the use of accuracy profile and total error concept to monitor the adequacy of the results obtained by the new prototype. The method comparison was based on the Bland and Altman approach by comparing results obtained by the low-cost CE and a conventional set-up. Subsequent validation studies were realized with neutral and acidic drug molecules, each focusing on a single concentration level calibration curve in order to maintain as low as possible the expenses due to reagents and thus the cost of analysis, as important advantages of CE for drug quality control.

1. Introduction

A counterfeit medicine is a medicine that is deliberately and fraudulently mislabelled with respect to identity and/or source [1–3]. Both branded and generic products can be concerned by the counterfeiting. Counterfeits may include products (i) with correct ingredients/components, (ii) with wrong ingredients/components, (iii) without active ingredients, (iv) with incorrect amounts of active ingredients or (v) with fake packaging [1,3]. The consequences of counterfeiting are mainly observed among patients, the medicines end-user's supposed to be the beneficiaries. They are exposed to risk of consuming such counterfeit products, i.e., therapeutic failure or drug resistance [4]. Some extreme cases can lead to death. Dramatic examples are reported in Panama and Nigeria. In 2006, more than 100 patients have been killed in Panama by medicines manufactured with counterfeit glycerine; in 2008, about 100 babies died because of absorbing a false paracetamol syrup [3,5]. The proportion of counterfeit medicines is dramatically increasing these days [6–9], in particular in some African countries pharmaceutical wholesalers where it was reported up to 80% of counterfeiting [10]. Unfortunately consumers and prescribers are unable to assess the quality, safety and efficacy of medical products.

Considerable efforts are deployed in order to truly fight and prevent trade in counterfeit medical products [1,3,7,10–13]. One of them is based on the dissemination of information useful for assessing technologies aimed at preventing, deterring, or detecting counterfeit medicinal products. This is somewhat paradoxical in particular for poor/emerging countries since such practices require adequate/large infrastructure or facilities, i.e. well equipped

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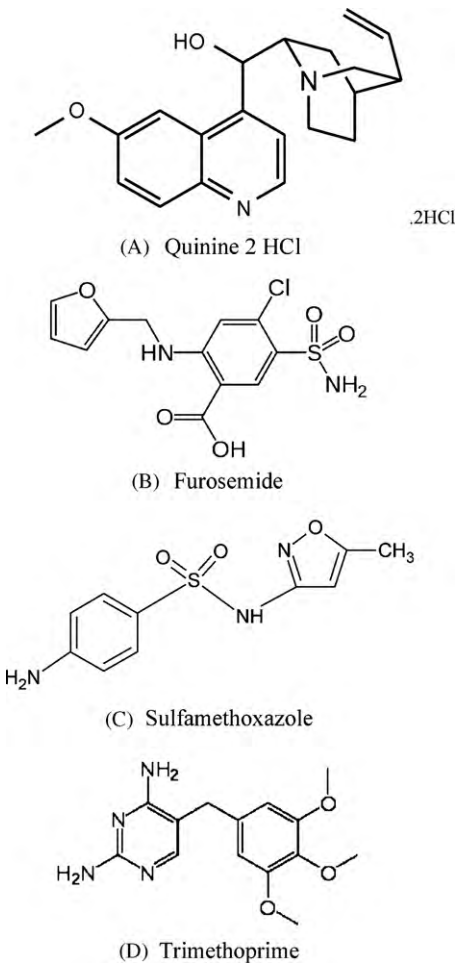

Fig. 1. Chemical structures of quinine dihydrochloride (pK_a = 8.5) (A), furosemide (B), sulfamethoxazole (C) and trimethoprime (D).

laboratory that is very often lacking or simply not functioning. Unfortunately, lack/insufficient of such facilities reduces the capacity of the Regulatory Agencies to react rapidly and adequately based on scientific considerations, thus leading to ineffective control. Several analytical methods, including near infrared spectroscopy, Raman spectroscopy, refractometry, colorimetry, X-ray powder diffraction analysis, nuclear magnetic resonance [14–22] and separations techniques such as liquid chromatography (LC), thin layer chromatography, gas chromatography and capillary electrophoresis (CE) [23–30] are used to analyse pharmaceutical substances, taking into account their variety of structure and chemical properties such as polarity and acidity. These methods need straightforward sample preparation and rapidity to support decisions in pharmaceutical fields, i.e., batch release or rejection, etc. However, for most of these techniques, financial expenses are not affordable to allow an easy implementation for a regular, systematic and wide-spread (or extended) control. Furthermore, the apparatus maintenance is one of the crucial issues allowing a long-term use of an analytical device in emerging countries.

Last 20 years, CE has gained importance for its ability to analyze several compounds with good selectivity [27,28]. Several aspects can largely contribute to the CE implementation in poor/emerging countries: simple, reliable and (cost-)efficient drug control methods, financial expenses, i.e., solvent and reference material consumption [31], water is often the solvent of choice, ease of operation (no need of complex solvent gradients [32]). Finally, several generic conditions have been reported for separating molecules. In this context, the University of Applied Sciences Western Switzerland, College of Engineering and Architecture of Fribourg (UAS-WS-FR) has developed in collaboration with the School of Pharmaceutical Sciences, University of Geneva-Lausanne and the Geneva University Hospital (HUG) a low cost analytical device, based on capillary electrophoresis (CE), with the aim to use it for educational purpose in developing and transitional countries. Three prototypes were built, the first one, within the period 2006–2007, where the mechanical and electronical issues were assessed, including an original detection device built on the basis of diode technology. The second, within the period 2007–2008, for the software and ergonomic optimisations and the third one (2009), was used for the development of methods of drugs analysis, selected toward a list submitted by African partners located in Mali, where it is now located. In the method development stage, in collaboration with the Institute of Pharmacie, University of Liège, a particular attention was paid to the robustness of the system in order to anticipate the problems that can be encountered when dealing with analytical methods in Africa as well as the quantitative aspects of quality controls of drugs.

In this paper, the analytical performance and the ability of the developed low-cost CE equipment for quantification is presented. A complete validation study with minimal requirement in regards to calibration purposes, were performed on representative drugs with different physico-chemical properties: quinine (QUN), furosemide (FUR) and the combination trimethoprime (TMP)/sulfamethoxazol (SMX) (see chemical structures in Fig. 1). These medicines represent one of the most targeted pharmacological groups by counterfeiting namely the antimalarial (21% of counterfeiting), diuretic (9%) and anti-effective (12%) drugs, respectively [35]. A strategy based on the total error of measurement was applied [33,34] and completed with a method comparison in order to evaluate the performance of the low-cost CE equipment to another one commonly available on the market.

2. Experimental

2.1. Instrumentation

Experiments for method development and validation of QUN were performed on an Agilent HP^{3D}CE system (Hewlett-Packard, Waldbronn, Germany) equipped with an on-column diode-array detector, an autosampler, a high-velocity air-cooled capillary cartridge, a power supply able to deliver up to 30 kV and an external pressure system. A CE ChemStation software version Rev. A.10.02 was used to control the CE instrument, to acquire and to handle the data. Separations were performed in bare fused-silica capillaries provided from Polymicro (Phoenix, AZ, USA).

Further experiments were carried out on a CE Budget Device Prototype 2 (ECB2) (HES-SO, Fribourg, Switzerland) [36,37]. This equipment consists of a CE device equipped with a detection system based on light-emitting diodes (LEDs) with a lamp at a single wavelength at 254 nm. The ECB2 (Fig. 2) is equipped with two manual samplers, one for inlets and one for outlets, a temperature sensor since there is no temperature control of the capillary. For the control of the instrument, the acquisition and the analysis of data, a “Chromatos” software developed at the HES-SO University was used.

The accuracy profiles as well as the statistical calculations including validation results and different uncertainty estimates were obtained using the e-noval software version 3.0 (Arlenda, Belgium). JMP software version 7.0 for Windows (SAS Institute, Cary, NC, USA) was also used for further statistical calculations.

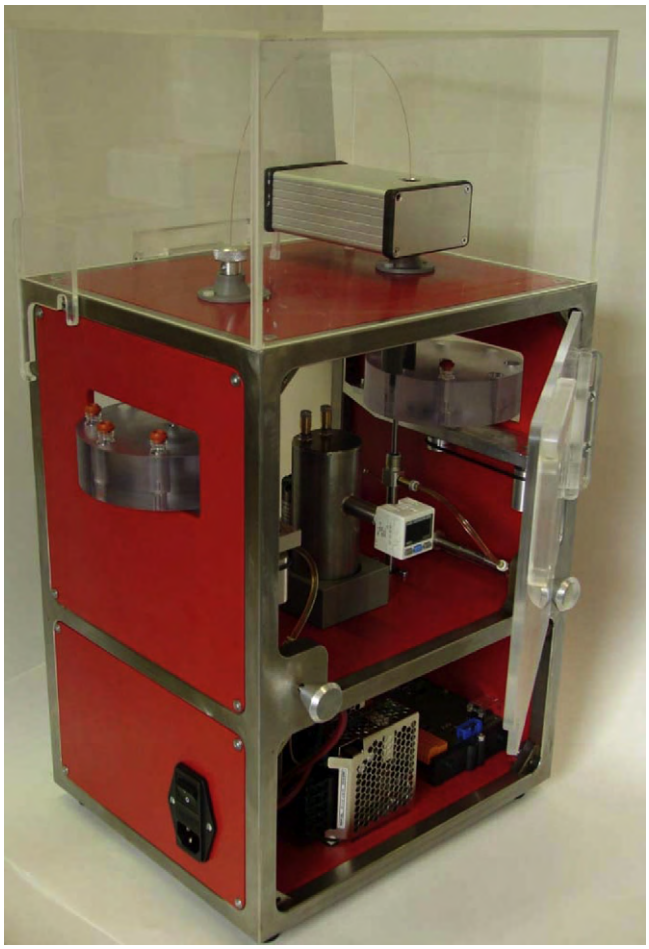

Fig. 2. Capillary electrophoresis budget device (ECB2).

2.2. Chemical and reagents

Samples of quinine dihydrochloride (QUN), furosemide (FUR), trimethoprime (TMP), sulfamethoxazole (SMX), procaine and phenobarbital were obtained from Sigma–Aldrich (St-Louis, MO, USA), respectively. Other substances tested as internal standards were obtained from several pharmaceutical companies. Sodium hydroxide, tri(hydroxymethyl)aminomethane, boric acid, acetic acid, hydrochloride acid and orthophosphoric acid were of analytical grade. Methanol of LC grade was purchased from Panreac (Barcelona, Spain) and ultrapure water was supplied by a Milli-Q gradient A10 purification unit from Millipore (Bedford, MA, USA).

2.3. Electrophoretic technique

For the HP^{3D}CE, a bare fused-silica capillary with 50 μm internal diameter and 32.5 cm total length (24.5 cm from inlet to the detection window, effective length) was used. The capillary was conditioned by flushing the following sequence: (1) methanol for 3 min, (2) water for 5 min, (3) NaOH 1 M solution for 3 min, (4) NaOH 0.1 M solution for 3 min, (5) water for 5 min, (6) HCl 0.1 M solution for 3 min, (7) water for 5 min and (8) background electrolyte (BGE) for 5 min. Each day before starting measurements the capillary was flushed with methanol, water and BGE for 5 min each, and after the run or before storing the capillary, it was flushed with methanol and air for 5 min each. Detection was performed at 254 nm with a band width of 10 nm and a reference signal at 426 nm (band width 100 nm). The choice of this wavelength was to make feasible any transfer method to the ECB2 equipment equipped with a LED fixed at 254 nm. The choice was also to allow a comparison of results considering as much as possible the same parameter values within the two equipments.

For the ECB2 equipment, a fused-silica capillary with 50 μm internal diameter and 51 cm total length (39.8 cm from inlet to the detection window, effective length) was used. The capillary was conditioned by flushing the same sequence as for Agilent HP^{3D}CE. Also the washing before and after measurements was done in the same way as for HP^{3D}CE. Detection was performed at 254 nm.

The different BGEs were phosphate buffer, borate buffer and acetate buffer prepared from orthophosphoric acid, boric acid and acetic acid, respectively.

Table 1
Preparation of standard solutions related to QUN, FUR, SMX and TMP.

Concentration level (%)	Concentration of QUN, FUR, SMX and TMP	
	Calibration standards (two independent series)	Validation standards (four independent series)
80	80 ppm[a]	80 ppm
100	100 ppm	100 ppm
120	120 ppm[a]	120 ppm

[a] Only for QUN.

2.4. Preparation of standard solutions

2.4.1. Validation

The dissolution of analytes was realized in a mixture of methanol:water (1:1) while dilutions were achieved in water. The number of series of validation was three for SUX and TMP, and four for QUN and FUR.

2.4.1.1. Solutions used for calibration. Different stock solutions were prepared by dissolving separately 400 mg of QUN 2HCl in 10 mL of methanol:water mixture (1:1), 20 mg of FUR in 2 mL, 400 mg of SUX in 5 mL and 80 mg of TMP in 5 mL. Subsequent dilutions were done in water to obtain intermediate and final solutions at the theoretical concentration levels given in Table 1. These solutions constitute the calibration standards (CS) prepared twice in order to have two independent standards. Each calibration solution was prepared in such a way to contain the selected internal standard at 100 ppm when analysing TMP or FUR, and at 200 ppm when analysing SUX or QUN.

2.4.1.2. Solutions used for validation. Independent stock solutions of QUN, FUR, TMP and SMX were prepared as mentioned in Section 2.4.1.1. Subsequent dilutions were carried out to obtain intermediate and final solutions at concentrations mentioned in Table 1. All these solutions were prepared and filled up to the final volume of 10 mL with a solution of D-manitol (10 mg/mL), the matrix simulating solution. The corresponding internal standard content was 100 ppm. These solutions constitute the validation standards (VS) prepared three times in order to have three independent standards, except for QUN which was prepared four times.

To check the selectivity of each method, several solutions were prepared and tested: blank (water), D-manitol at 10,000 ppm, individual solutions of each internal standard at 100 ppm, individual solutions of each compound to analyse at 100 ppm, mixture solutions containing 100 ppm of the analysed compound and its corresponding internal standard at 100 ppm.

2.4.2. Routine analyses

2.4.2.1. Reference sample solution. The reference solutions were prepared as mentioned in Section 2.4.1.1.

Table 2
Experimental domain for the tested factors applied to univariate optimization of the CE method.

Type of factor	Antidiuretic compounds				Anti-effective compounds				Antimalarial compounds			
pH levels	Level 1 8.5	Level 2 8.8	Level 3 9.0	–	Level 1 6.1	Level 2 6.2	Level 3 6.9	–	Level 1 2.5			
Type of buffer for BGE	Type 1 Boric acid				Type 1 Acetic acid		Type 2 Orthophosphoric acid		Type 1 Boric acid		Type 2 Orthophosphoric acid	
Concentration of buffer for BGE (mM)	Level 1 25 mM	Level 2 50 mM	Level 3 75 mM	Level 4 100 mM	Level 1 25 mM	Level 2 50 mM	Level 3 75 mM	Level 4 100 mM	Level 1 25 mM	Level 2 50 mM	Level 3 75 mM	Level 4 100 mM
Counterion	Type 1 Tris-BGE		Type 2 Na-BGE		Type 1 Tris-BGE		Type 2 Na-BGE		Type 1 Tris-BGE		Type 2 Na-BGE	

2.4.2.2. Test sample solution. Sample preparations were different for the two types of matrix considered, the liquid or the solid one.

2.4.2.2.1. Solutions from liquid matrix. For QUN, FUR and cotrimoxazole (SMX + TMP), the test sample solutions were prepared from injectable solutions of 2 mL to 5 mL. Subsequent dilutions were done with water to obtain a final concentration of 100 ppm of each original compound. The last dilution was done in such a way to contain 100 ppm of the corresponding internal standard.

2.4.2.2.2. Test sample solution for solid matrix. Solid matrix (pills) needed some more precautions to ensure sufficient extraction of the active ingredients. Three pills accurately weighted were pulverized and mixed with a mortar. From the pulverized powder, an amount corresponding to a pill average weight was quantitatively transferred in a 10 mL volumetric flask. Methanol was added to the mark and the mixture was shacked mechanically for 10 min. Then, the extract solution was filtered with a Polyester Chromafil PET-45/25 filter (0.45 μm). Subsequently a series of dilutions were done with water to obtain a final concentration of 100 ppm of the original compound and containing 100 ppm of the corresponding internal standard.

3. Results and discussion

3.1. CE method development

In this study, CE methods were developed for identification of counterfeit drugs, with a focus on the determination of the presence of the active ingredient and the possibility to rapidly decipher regarding the correct amount of the active ingredient. Thus, several aspects needed to be taken into account: (i) simple and generic methods, in order to analyze a high number of compounds and make easier the method selection for each analyte with basic chemistry knowledge; (ii) low cost methods, based on low solvent consumption and no special sample preparation equipment; and (iii) ECB2 characteristics: UV detection based on LED technology at 254 nm, maximum voltage during the separation at 20 kV to avoid Joule effects and injection at 50 mbar.

The analytical conditions were initially investigated on the commercially available device, then transferred with parameters adjustments to the ECB2 for keeping selectivity constant, such as the injection conditions in order to maintain constant the injection volume, the applied voltage and electric field. For the generic method development, drugs were divided into three groups according to their pharmacological use: antimalarial compound (QUN), diuretic compound (FUR) and anti-effective drug (cotrimoxazole: mixture 1:5 of TMP:SMX). For each group, several factors were tested: pH of BGE, type and concentration of buffer for the BGE, counterion (Table 2) and postplug. During experiments, the identity of the peak signal for each component of interest as well as the IS was achieved by mean of injection of individual sample solution and by means of UV–Vis spectrum extracted from the diode-array detector.

3.1.1. Antimalarial compounds

Considering QUN with its pK_a (5 and 9.1), the suitable pH value for the analysis was 2.5 since at this pH this compound is doubly ionized allowing its cathodic detection. In addition, this value is a well known starting point when performing analyses of basic compounds in acidic conditions with a phosphate buffer (pK_a is 2.12) as BGE. The latter was set at 50 mM to allow a good buffering power (buffering range 1.6–3.2) with good selectivity and moderate power production. Procaine HCl (pK_a = 8.9) was used as internal standard (IS). Two types of counterion were evaluated with phosphate, sodium hydroxide (Na^+ counterion) and Tris(hydroxymethyl) aminomethane ($Tris^+$ counterion). With Tris-BGE, the peak of QUN and IS were narrower with less tailing than with Na-BGE (data not shown) and was attributed to the better mobility matching of the analytes in presence of $Tris^+$ vs Na^+. Furthermore, a lower current was observed with Tris-BGE permitting the use of higher voltage to gain resolution. The CE method was then transferred on the ECB2 and tests were performed using 50 mbar for 8 s and CE parameters were adapted as shown in Table 3.

3.1.2. Diuretic compounds

The BGE selected was boric acid (pK_a at 9.2) that gives a good buffering capacity around this pH range. At first, salicylic acid, ibuprofen and phenobarbital were tested as IS under the initial CE conditions according to criteria described in the literature [38–40], in particular, a comparable absorptivity at 254 nm, the fixed wavelength of LED. Salicylic acid and ibuprofen were found to co-migrate with FUR. Phenobarbital was appropriate, cheap and easily available. After evaluation of the pH value in the range 8.5–9.0, pH 8.8 was selected. It has to be noted that FUR was well separated from the IS with an acceptable analysis time whatever the values of pH tested, which could therefore be considered as a robust parameter. Regarding the buffer concentration, the best resolution was observed at 100 mM, with good peak symmetry and sufficient peak height. The current was found acceptable to avoid any Joule effect,

Table 3
Capillary electrophoresis parameters used for the analysis of the QUN compounds in the ECB2 and the Agilent HP^{3D}CE.

Parameters	Units	ECB	Agilent HP^{3D}CE
Capillary			
Type	–	TSU fused silica	TSU fused silica
Internal diameter	μm	50	50
Total length	cm	51	32.5
Effective length	cm	39.2	24.5
Voltage	kV	20	13
Electric field	V/cm	392	400
Temperature	°C	No control	25
Cleaning			
Between standards	min	1	1
Between measurements	min	3	3

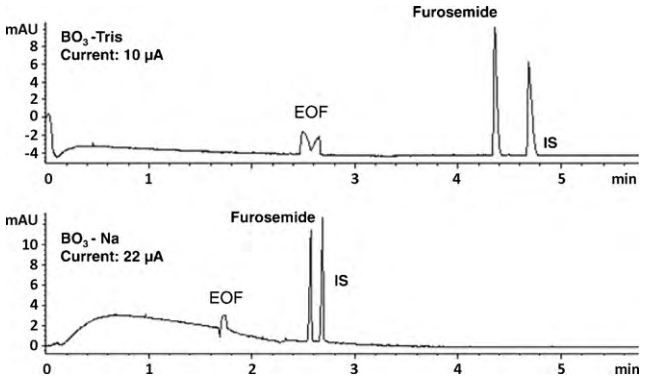

Fig. 3. Effect of BGE counterion on EOF velocity, current and separation of FUR and phenobarbital.

Table 4
Method parameters for the analysis of FUR, TMP and SMX, in the ECB2.

Parameter	Unit	Value for FUR	Value for TMP and SMX
Capillary			
ID	µm	50	50
$^{+}L_T$	cm	51	51
$^{++}L_d$	cm	39.2	39.2
Voltage	kV	20	20
Ramp	kV/s	1	1
E	V/cm	400	392
Injection			
Time	s	10	8
Pressure	mbar	75	50
BGE			
Type		Tris-Borate	Tris-Phosphate
Concentration	mM	100	50
pH		8.8	6.1

while maintaining an acceptable analysis time. Finally, the counterion effect was tested. Fig. 3 shows that a better peak separation was obtained with Tris–Borate. Indeed, Tris+ showed an important effect on reducing the EOF more likely due better mobility matching. Thus, the final BGE was a 100 mM Tris–Borate buffer. The method was then transferred to ECB2 with the parameters presented in Table 4.

3.1.3. Anti-effective drugs

Cotrimoxazole was selected since it contains a synergistic mixture of two compounds, TMP (pK_a of 7.3) and SMX (pK_a of 5.7). The tested pH range was almost neutral (see Table 2). It can be noticed that these compounds are in the ratio of 1–5 in the formulation. Tests were thus performed in order to determine the appropriate dilution of the formulation allowing the simultaneous analysis of both compounds with Procaine HCl (100 ppm) used as IS. After investigation, an almost neutral BGE was found to be appropriate to analyse TMP as a cation and SMX as anion. Analyses were first performed with acetate buffer (pK_a = 4.75) and finally, 50 mM of phosphate–Tris buffer at pH 6.1 (50 mM) was selected since it gave good peak selectivity and short analysis as shown in Fig. 4 where the sample had a composition of 40 ppm of TMP and 200 ppm of SMX. TMP and Procaine are basic compounds and appeared before the EOF, while SMX (amphoteric) migrated after the EOF in the selected conditions. The final method parameters are presented in Table 4.

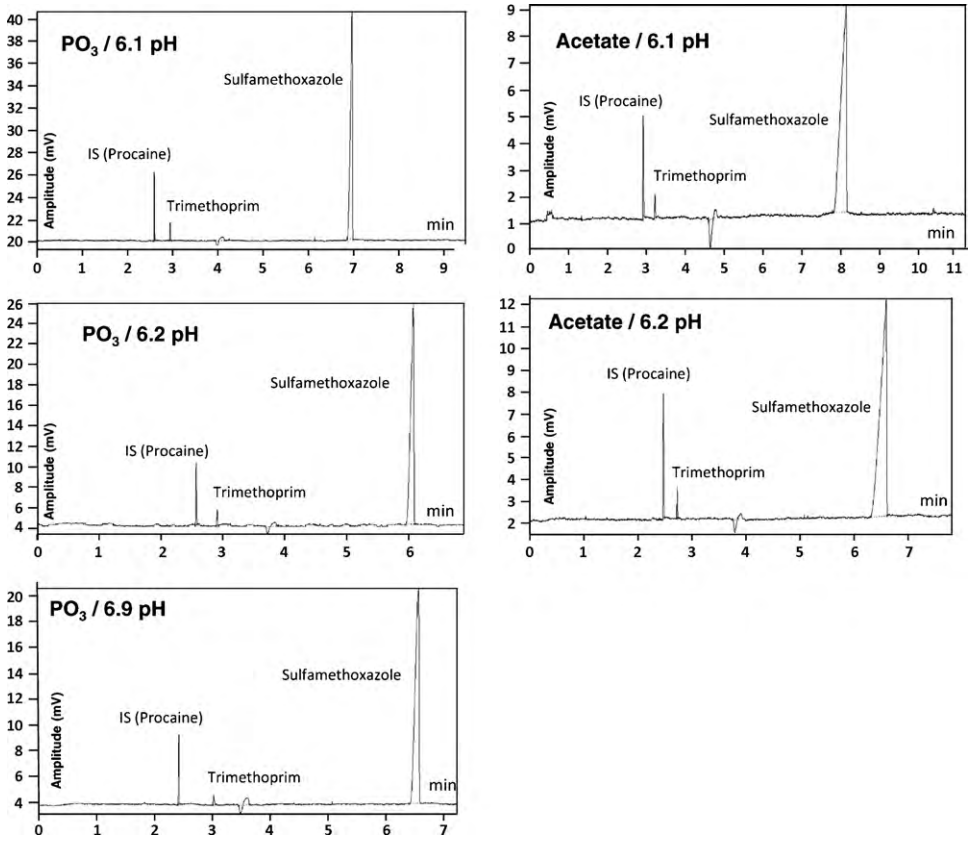

Fig. 4. Effect of pH on the separation of SMX and TMP and procaine used as IS. *Sample solution*: A solution containing 40 ppm of TMP, 200 ppm of SMX and 100 ppm procaine.

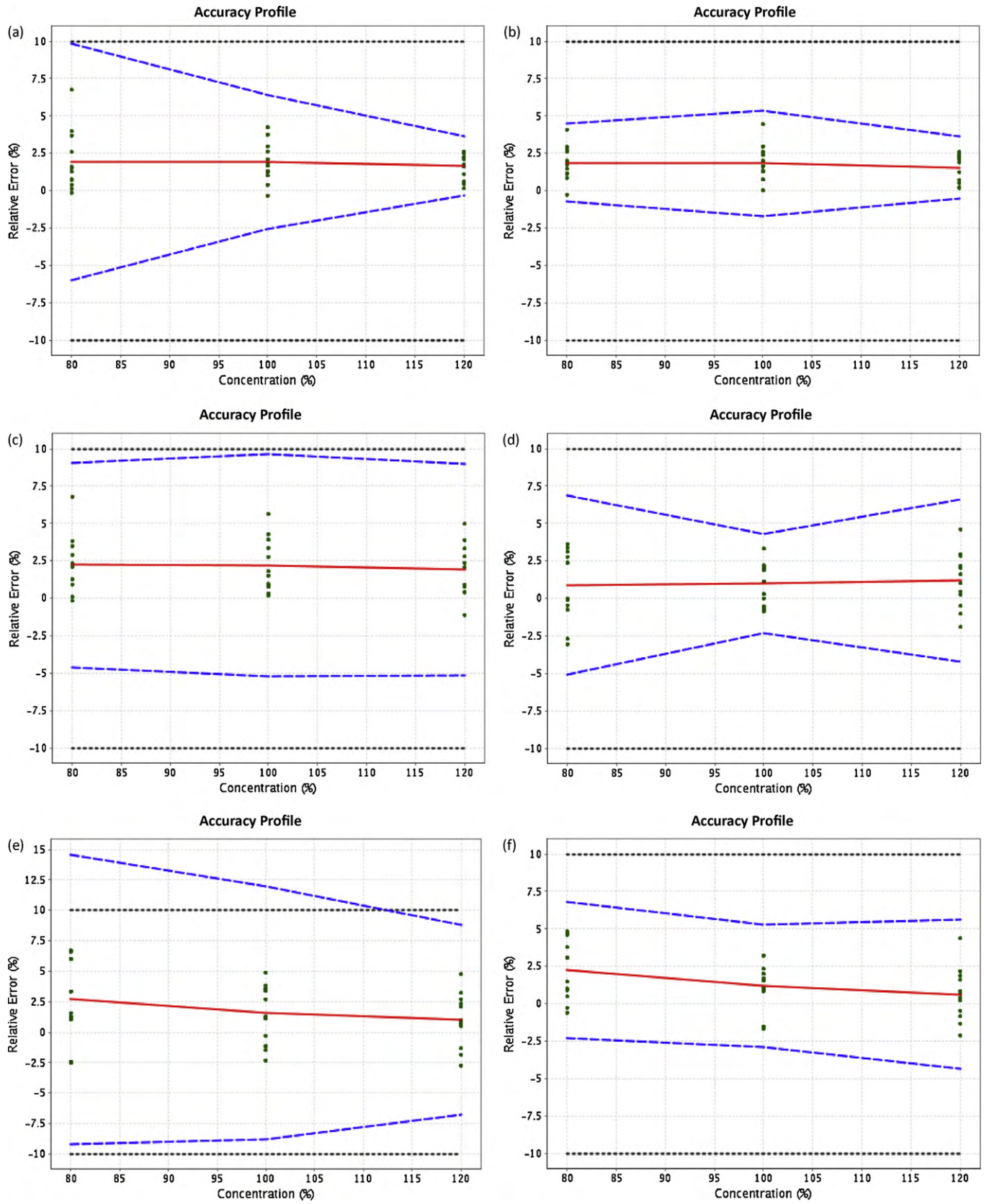

Fig. 5. Accuracy profiles of QUN. Agilent equipment: accuracy profiles obtained by considering: (a) a simple linear regression model, (b) a linear regression model through zero fitted with the maximum level of concentration (120%) and (c) a linear regression model through zero fitted with the medium concentration level (100%). Prototype ECB2 equipment: accuracy profiles obtained by considering: (d) a simple linear regression model, (e) a linear regression model through zero fitted with the maximum level of concentration (120%) and (f) a linear regression model through zero fitted with the medium concentration level (100%). *Plain line*: relative bias; *dashed lines*: relative β-expectation tolerance limits (β = 95%); *dotted curves*: acceptance limit (±10%); and *dots*: relative back-calculated concentrations of the validation standards.

3.2. Validation

Accuracy profiles based on tolerance intervals for the estimation of total error was applied to demonstrate the assays results adequacy [33,34] on the original ECB2 apparatus. The tolerance interval used is a "β-expectation tolerance interval" defining an interval in which it is expected that each future result has a probability (β) to fall [41–43]. It is therefore a predictive methodology. This tolerance interval was computed for each concentration level tested, using their estimated intermediate precision standard deviation and bias. By joining the upper tolerance limits on the one hand and the lower tolerance limits on the other hand, it defines an accuracy profile. As long as this profile stays inside predefined acceptance limits the method can be considered as valid. Indeed, it guarantees that each future result will be included in the a priori set acceptance limits with at least a probability β (e.g. 0.95 or 95%). Such an approach reflects more directly the performance of individual assays than other methodologies [44]. The concept of accuracy profile was also used to select the most appropriate regression model for calibration and the range over which the method can be considered as valid. The acceptance limits were all settled at ±10% according to the final aim of each electrophoretic method which is the quantitative determination of active substances of drug products and further discussion with the African partner in case of quantitative drug analysis. The minimum probability to have results included within these acceptance limits was set at β = 95%. This means that, to declare the methods as valid, each future result must have at least 95% chance to fall within the acceptance limits of ±10% total error [34,35,40–43].

3.2.1. Quinine determination

3.2.1.1. Methods validation. The two different equipments (commercially available and prototype) were used to validate the method. For each validation, three types of calibration model were evaluated to find the simplest and most adequate one to achieve the objective of the method. Based on simple linear regression, the three calibration model were a complete external calibration using all the concentration levels of the calibration standards, a linear regression forced through the origin with the highest concentration level of the calibration standard (120%) and a linear regression forced through the origin and the target (medium) concentration level of the calibration standard (100%).

For each equipment and using each of the response function tested, accuracy profiles were obtained and compared in order to (1) compare the results accuracy obtained over the range of concentration tested and (2) select the most appropriate regression model for calibration curve. The six accuracy profiles obtained are given in Fig. 5A–F. For the commercial device (Fig. 5A–C) all the regression models tested allowed to obtain accurate results. Indeed the relative tolerance limits are all included inside the acceptance limits of ±10%. However, in order to integrate practical efficiency with adequate results quality, the linear regression forced through zero using the 120% calibration level was selected. This calibration curve is the simplest model that provides accurate results. Furthermore, this model allows reducing the imprecision of the results as depicted in Fig. 5C: the distance between the lower and upper relative tolerance limits is the smallest. This distance, for a constant sample size and probability level, is only dependant on the between and within series variance components. The accuracy profiles obtained with the prototype are illustrated in Fig. 5D–F for the three regression models tested and demonstrate that the simple linear model and the forced through zero using the 100% calibration level provided accurate results. With similar requirements than for the Agilent device, the retained calibration curve was the forced through zero using the 100% calibration level (target concentration) regression model. Table 5 summarizes for each apparatus, the validation performance criteria of the method: trueness, precision, accuracy, range and linearity as required by ICHQ2 guideline [45]. It can be noticed a positive relative bias which is fairly constant over the concentration range for the commercial one and that decreases when the concentration increases for ECB2. This was confirmed by the linearity information, where for both equipment the intercept is a positive value. For the commercial equipment the slope is nearly close to one, confirming also the absence of proportional bias, whereas for the ECB2 one, the slope is smaller than 1 showing that the bias decreases when the concentration increases. Concerning methods precision, one striking element is the nearly absence of day-to-day variability with both technology. The intermediate precision RSD never exceeds 2% whatever the equipment and irrespective of the concentration level.

Table 5
Validation results obtained for the validation of the method dedicated to the quantification of QUN by the Agilent HP^{3D}CE and the prototype ECB2 electrophoretic equipments, using a regression forced through zero using the highest concentration level of the calibration standards (120%) and a regression forced through zero using the medium concentration level of the calibration standards (100%), respectively.

Validation criterion	Relative bias (%)	
	Agilent	ECB
Trueness		
80%	1.90	2.3
100%	1.80	1.2
120%	1.60	0.6
Validation criterion	**Repeatability/intermediate precision RSD (%)**	
	Agilent	ECB
Precision		
80%	1.1/1.1	2.0/2.0
100%	0.9/1.3	1.2/1.5
120%	0.9/0.9	1.4/1.9
Validation criterion	**Relative Tolerance limits (%)**	
	Agilent	ECB
Accuracy		
80%	[−0.7; 4.5]	[−2.3;6.8]
100%	[−1.7; 5.4]	[−2.9; 5.3]
120%	[−0.7; 3.7]	[−4.4; 5.6]
Linearity		
Range (%)	80–120	80–120
Slope	1.009	0.9733
Intercept	0.7968	3.908
r^2	0.9962	0.9892

3.2.1.2. Methods comparison. A direct comparison of the results obtained by the two equipments was made using the previously validated methods. The Bland–Altman approach was used [46], taking as maximum relative difference between the results obtained by both equipments a limit of ±10%. Fig. 6 shows the difference plot obtained. It can be seen that the 95% limits of agreement do not exceed ±5%, that is 95% of the results differences between the two apparatus are included within these limits. It is thus evident that the two equipments gave comparable results.

3.2.2. Other methods validation using the prototype device

The two other methods, for FUR and the simultaneous determination of TMP and SMX were validated. The validation protocols used were designed with a single point calibration at the target value (100% level) for the calibration curves according to the previous results and with the aim to obtain the simplest, rapid and efficient method that provide accurate results. The analytical objectives were identical with acceptance limits set

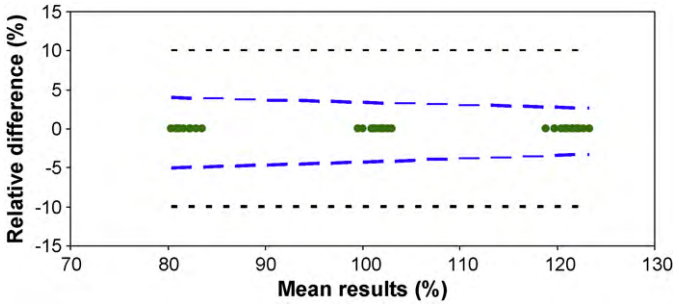

Fig. 6. Bland and Altman plot of the relative differences of the results obtained by the Agilent and the prototype ECB2 equipment versus the mean results of the two equipments. *Dashed lines*: 95% agreement limits of the relative differences; *dotted lines*: maximum acceptable relative difference between the two equipments set at ±10%; dots: relative differences.

at ±10% for the three compounds and the probability β at 95%.

3.2.2.1. Furosemide determination. The accuracy profile and validation results for the quantification of FUR are shown in Fig. 7A and Table 6, respectively. From this figure, it can be seen that the method provides accurate results over the whole concentration range tested. Table 6 shows that the relative bias and the intermediate precision never exceeded 2.0%. Furthermore, only a small increase in the results variability due to the day-to-day variability was observed as shown by the small difference between the repeatability and intermediate precision RSDs.

3.2.2.2. Trimethoprim determination. Accurate results for the method dedicated to the quantification of TMP were also obtained over the whole concentration range tested as shown by the accuracy profile in Fig. 7B. Indeed the 95% relative tolerance limits were all fully included inside the symmetric acceptance limits of maximum 10% total error. Trueness and precision of the method did not exceed 2% as shown in Table 6. Furthermore, only repeatability sources of variability influence the dispersion of the results.

3.2.2.3. Sulfamethoxazol determination. The accuracy profile obtained for the quantification of SMX in Fig. 7C, shows that the method was not able to provide accurate results at the 80% concentration level as the 95% relative tolerance limits exceed

Table 6
Validation results obtained for the validation of the method dedicated to the quantification of FUR, TMP and SMX using the prototype ECB2 equipment.

Validation criterion	Compounds tested		
	FUR	TMP	SMX
Trueness (relative bias, %)			
80%	1.3	1.9	−2.3
100%	1.2	1.6	−1.9
120%	1.9	0.8	−1.6
Precision (repeatability, %RSD/intermediate precision, %RSD)			
80%	1.2/1.2	1.8/1.8	1.7/2.5
100%	1.2/1.7	1.2/1.2	2.1/2.1
120%	1.6/1.7	0.8/0.8	1.6/1.8
Accuracy (relative limits, %)			
80%	[−1.5; 4.2]	[−2.6; 6.3]	[−13.6; 8.9]
100%	[−3.6; 6.1]	[−1.5; 4.6]	[−7.1; 3.3]
120%	[−2.0; 6.0]	[−1.1; 2.8]	[−6.1; 3.0]
Linearity			
Range (%)	80–120	80–120	80–120
Slope	1.032	0.988	0.9998
Intercept	−1.633	2.564	−1.857
r^2	0.9917	0.9948	0.9873

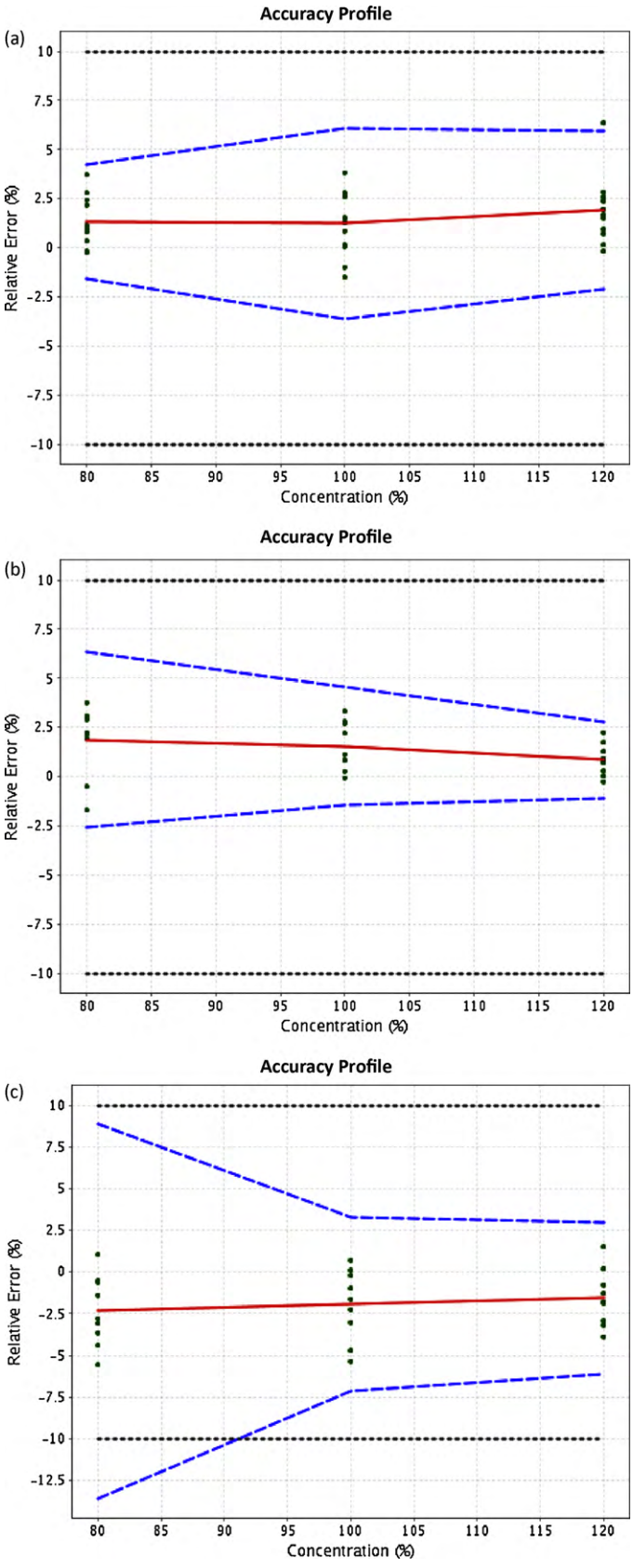

Fig. 7. Accuracy profiles obtained for the methods dedicated to the determination of (a) FUR, (b) TMP and (c) SMX. *Plain line*: relative bias; *dashed lines*: relative β-expectation tolerance limits (β = 95%); *dotted curves*: acceptance limit (±10%); and *dots*: relative back-calculated concentrations of the validation standards.

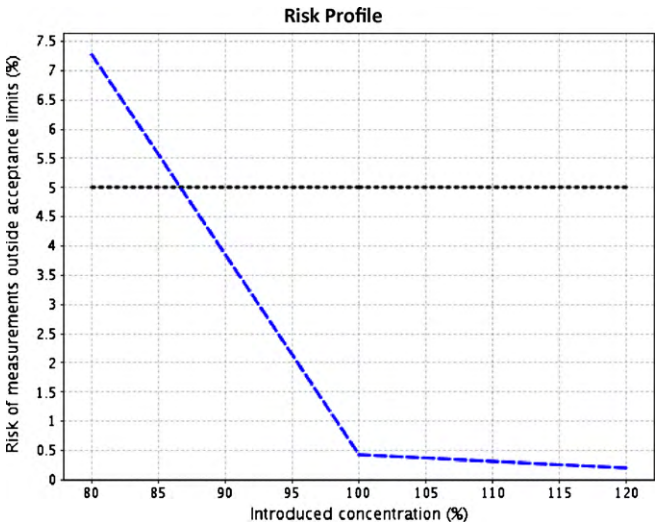

Fig. 8. Risk profile of the method dedicated to the quantification of SMX. *Dotted line*: maximum risk of 5%; *dashed line*: effective risk of having results falling outside the specified acceptance limits set at ±10%.

Table 7
Contents of QUN and FUR in suspect samples.

Compound	Matrix	Experimental concentration (ppm)	Theoretical concentration (ppm)
Quinine	Liquid	90	100
	Solid	75	100
Furosemide	Liquid	95	100
	Solid	95	100

the acceptance limits of ±10%. Indeed, Table 6 shows that, at this concentration level, the lower 95% relative tolerance limit was about −13.6%. This table also shows that for this concentration level, the bias is the strongest (−2.3%) as well as the intermediate precision RSD (2.5%). Furthermore it can be seen that the day-to-day variability is amplified relative to the other concentration level as shown by the larger discrepancy between the repeatability and intermediate precision RSD values. With a risk value to obtain result outside the acceptance limits of ±10% of 7.3% (Fig. 8), the method was considered valid throughout the completed investigation range [47].

3.3. Application

The suitability of the developed and validated CE methods to target potential counterfeit drugs was confirmed by quantifying QUN and FUR in commercially available injectables and pills products obtained from Mali. For furosemide, tablets of 40 mg (furosemide tablets 40 mg, Sino Pharma S.A. Mali, RC Sanke, Bamako, Mali) and injectable solution containing 20 mg (Lasix, Sanofi Aventis, Meyrin, Suisse) were used. For Quinine, film-coated tablets containing 300 mg of quinine sulphate (Remedica, Limassol-Cyprus, Europe) and injectable solution of quinine dihydrochloride (no trademark available, Batch No. 070820, Exp 08/2010) were used. The ECB2 equipment was employed and the regression models selected in the methods validation were employed. Table 7 gives the content of QUN and FUR obtained for the two types of product. As can be seen in this table, content of QUN and FUR in injectable formulation were comprised within the acceptance limits of 10%. However Table 7 shows that for the solid formulation the content of QUN is far below these acceptance limits suggesting thus a counterfeit drug.

4. Conclusion

Drug counterfeits is a major public health issue in some poor and emerging countries. In order to fight this problem a robust and low cost analytical device was developed. Three methods were developed for the quantitative analysis of active substances present in pharmaceutical formulations subjected to be counterfeited and selected according to their therapeutic uses. For these methods, among the counterion tested, Tris+ gave the best results. Suitable parameter values were obtained allowing the CE separation of QUN, FUR and SMX + FUR with their corresponding IS. The methods were applied with the low-cost CE equipment and results of validation, comparison study with an available CE equipment and routine quantification confirmed the performance of this low-cost CE device which can be easily implemented in poor and emerging countries.

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