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An LC method for the simultaneous screening of some common counterfeit and sub-standard antibiotics Validation and uncertainty estimation

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Abstract

Pharmaceutical counterfeiting is a worldwide public health problem, often under-recognised, especially in developing countries where the percentage of counterfeit and sub-standard medicines is dramatically high. Antibiotics, among the most widespread drugs, have been particularly targeted by counterfeiters. World Health Organization emphasizes the need for development and distribution of screening methods explicitly targeted to counterfeit drugs. In this paper is presented a single method for the simultaneous analysis of some of the most common and counterfeited essential antibiotics: ampicillin, amoxicillin + clavulanic acid, doxycycline, cloxacillin, chloramphenicol. A full validation was performed in terms of linearity, precision, robustness and trueness; an assessment of uncertainty was carried out exploiting these data. A wide linearity range was investigated considering the specific nature of counterfeit and sub-standard drugs, whose content in active substance may be rather far from the declared amount. A large span in robustness parameters was considered and a complete intermediate precision assessment was conducted, envisaging the possibility of transferring the method to quality control laboratories, hopefully in developing countries.

Finally, the method was successfully applied to the analysis of antibiotics purchased on the informal market in Chad, among which counterfeit and sub-standard samples were detected.

Keywords: Counterfeit drugs; Sub-standard drugs; Liquid chromatography; Screening method; Antibiotics; Uncertainty of measurement

1. Introduction

The phenomenon of production and sale of counterfeit medicines is increasing worldwide, representing a serious risk for public health. Although precise and detailed data on counterfeit medicines are difficult to obtain, estimates range from around 1% of sales in developed countries to over 10% in developing countries, depending on the geographical area [1–3]. Data reported in the Matrix of Drug Quality Reports by the U.S. Pharmacopeia [4] indicate that in some areas of Sub-Saharan Africa, South East Asia and Latin America counterfeits make up more than 30% of medicines. Illegal Internet sales are 50% fakes [3–4]. In developing countries pharmaceutical counterfeiting mainly concerns life-saving medicines such as antibiotics, antimalarials, anti-tubercular and antiretroviral drugs. In many cases counterfeiting consists in the absence of active substance, in the presence of a low quantity of active substance or in the substitution of the declared active ingredient with a cheaper one [5]. Some of the causes of the large diffusion of pharmaceutical counterfeiting in developing countries are lack of import controls and poor quality control on medicinal products at different levels of the distribution chain (import, wholesalers, official and informal vendors). A medicines quality control laboratory requires technology, high-specialised personnel and consistent funding, seldom available in less developed countries. Sometimes they succeed in affording the high cost of such a structure, but lack specific expertise for developing analytical methods for pharmaceutical counterfeiting detection. In this scenario, the need for simple liquid chromatographic screening methods is pressing.

Most of the published studies on analytical methods for counterfeit drug analysis propose two approaches: the development of very simple methods (e.g. colorimetric reactions or thin layer

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chromatography) [6,7] or complex but expensive methods (e.g. X-ray diffraction, Near Infrared Spectroscopy, Nuclear Magnetic Resonance [8–10]). Official Quality Control Laboratories in developing countries rarely have financial resources to buy expensive technologies, whereas can afford LC equipments. Moreover, the analysis of medicinal products according to official pharmacopeial methods calls for the application of different methods for each active substance: this is quite difficult when many different samples need to be screened. Thus in line with the strategies against pharmaceutical counterfeiting stated by WHO in the Declaration of Rome [11], in this paper a single method for the simultaneous analysis of some of the most common and counterfeited essential antibiotics is proposed. Over the last few years some LC methods for the simultaneous screening of potentially counterfeit medicines appeared in the scientific literature [12–15]. These papers deal with antimalarials, cephalosporins, anti-diabetic drugs, isometadinium products etc. To the authors' knowledge no method concerning the simultaneous analysis of the antibiotics discussed in this work has been published to date.

The developed method allows to separate six antibiotics in a single chromatographic run: ampicillin, amoxicillin + clavulanic acid, doxycycline, cloxacillin, chloramphenicol. Such substances were chosen taking into account both the high worldwide incidence of counterfeit antibiotic formulations [16] and their presence in the WHO Model List of Essential Medicines [17]. A full validation was performed in terms of linearity, precision, robustness, and trueness on products available in tablets or capsules (ampicillin, amoxicillin + clavulanic acid and doxycycline) while only linearity studies were performed for cloxacillin and chloramphenicol because of some difficulties in finding a suitable amount of products on the Italian market.

Validation data were also employed for measurement uncertainty estimation. Validation protocol was developed in view of the specific problem of pharmaceutical counterfeiting and of the possibility of transferring the method to quality control laboratories, hopefully in developing countries.

Finally, the method was successfully applied to the analysis of antibiotics purchased on the informal market in Chad.

2. Experimental

2.1. Chemicals

Ampicillin sodium salt (99.0%), cloxacillin sodium salt (98.0%), and chloramphenicol (99.0%) were purchased from Fluka (Sigma–Aldrich, Switzerland), doxycycline hyclate (99.0%) and amoxicillin (97.0%) were purchased from Sigma (Sigma–Aldrich) and lithium clavulanate (98.8%) was purchased from European Pharmacopoeia (EDQM, France). Potassium dihydrogen phosphate was from ICN Biomedicals Inc. (Ohio, USA), phosphoric acid 85% was from Friedel-de Haen GmbH (Germany). HPLC-grade methanol was from Baker (The Netherlands). All other reagents were of analytical grade. Medicinal products in tablets and capsules, containing amoxicillin + clavulanic acid (875 mg + 125 mg), ampicillin (500 mg) and doxycycline (100 mg), used in validation studies were purchased from the Italian National Market.

Suspected medicinal products containing amoxicillin, ampicillin and doxycycline in tablets and capsules were obtained on the informal market in Chad.

2.2. Standard and sample solutions preparation

Sample solutions were prepared in water at a final concentration of 0.1 mg/ml in active substance: 10 tablets of commercial medicinal products were powdered (for capsules, the content of 10 capsules was mixed) and a quantity of powder equivalent to 100 mg of active ingredient was weighed, quantitatively transferred to a 100 ml volumetric flask and brought to volume with water. Samples were sonicated for 10 min and then centrifuged (10 min at 3500 rpm – G-force 958 × *g*). Solutions were then filtered through 0.45 μm filters (Millex HV – Millipore). One millilitres of the filtered solution was diluted to 10 ml with water.

Standard solutions were prepared in triplicate by diluting a suitable amount of active substance in water to obtain a concentration of 1 mg/ml. Solutions were sonicated for 10 min and diluted with water to a final concentration of 0.1 mg/ml. Standard and sample solutions were analysed within 24 h. In this time interval solution stability at 2–8 °C and at room temperature was checked.

2.3. Chromatographic analysis

The LC system consisted of an Agilent 1100 chromatographic apparatus equipped with an automatic injector and a photo-diode array detector (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Data acquisition was performed by the Chemstation software (Agilent Technologies).

Analyses were carried out at 230 nm on a Zorbax SB C18 reversed phase column, 250 mm × 4.6 mm i.d., 5 μm particle size (CPS Analitica) at 25 °C. A Symmetry C18 column, 250 mm × 4.6 mm i.d., 5 μm particle size (Waters) was used for robustness studies only. Mobile phase A consisted of 10 mM potassium phosphate buffer (pH 5.1)/methanol, 95:5 (v/v) and mobile phase B consisted of 10 mM potassium phosphate buffer (pH 5.1)/methanol, 5:95 (v/v). Mobile phases were delivered at 1 ml/min by gradient elution from 5% B to 80% B in 23 min. The system was then re-adjusted to 5% B in 2 min and reconditioned for 5 min before the next analysis.

2.4. Method validation

2.4.1. System suitability

A System Suitability Test was performed on a solution containing amoxicillin, clavulanic acid, ampicillin, doxycycline, cloxacillin and chloramphenicol at 0.1 mg/ml each in water. The acceptance criteria were: areas and retention times RSD% < 1.0 for each analyte (six-replicated injections) and baseline separation of all peaks (resolution between chloramphenicol and doxycycline >3 and resolution between doxycycline and cloxacillin >3).

2.4.2. Specificity

Specificity was evaluated verifying the absence of chromatographic interferences with excipients, principal impurities and degradation products.

2.4.3. Linearity

Considering the specific application of this method to the screening of potentially counterfeit or sub-standard medicines, whose real content of active substance could considerably deviate from the declared amount, linearity was evaluated in a wide range. For each analyte it was determined on nine standard solutions from 10 to 150% of the test concentration (0.1 mg/ml).

2.4.4. Quantitation and detection limits

According to International Conference of Harmonization Guideline Q2 (R1) [18] quantitation and detection limits (LOQ and LOD) were not evaluated because they are not required for potency analysis of pharmaceuticals. The test concentration and the linear range were chosen far from the expected LOQ and LOD.

2.4.5. Uncertainty assessment

Most of the information needed for uncertainty determination was collected from precision and trueness studies. The remaining sources of uncertainty were investigated through robustness evaluation.

Precision, trueness and robustness studies provided uncertainty components in form of relative standard deviations, indicated as $U_{\text{precision}}$, U_{trueness} and $U_{\text{robustness}}$. All these components were then combined to obtain a relative *combined standard uncertainty* U_{c}:

$$U_{\text{C}} = \sqrt{U_{\text{precision}}^2 + U_{\text{trueness}}^2 + U_{\text{robustness}}^2}$$

A detailed description of the steps followed for uncertainty estimation, together with formulas and calculations are not reported in this paper since detailed procedures can be found elsewhere [19–33]. Only a schematic description will be given in the following paragraphs. Calculations were performed using an Excel spreadsheet and Statgraphics Centurion XV [34].

2.4.6. Precision studies and evaluation of $U_{precision}$

Precision component of uncertainty (i.e. the uncertainty due to method imprecision) was determined as follows. A 4-factor different intermediate precision was determined, the 4 factors being operator, equipment, time and random error. In particular a 4-factor nested experimental design was used. This planning is recommended by ISO [35–36] and has been extensively described and tested by various authors [26–28,37]. The schematic layout of the design is given in [26]. Samples were analysed by two operators, each operator performed, on each of two sets of equipment (comprising two HPLCs, two balances, two laboratory glasses sets, two reagents batches etc) six replicated determinations in each of 5 different days. As suggested in [26], the two equipments were not operated on the same day to avoid an underestimation of the day effect. In each analysis performed by each operator, a new standard solution was prepared in triplicate. Results were expressed as a percentage of the labelled amount. Outliers were determined by Grubb's test and deleted, while stragglers were retained. Analysis of variance (ANOVA) was used to obtain variance components from the four factors explored. In [35] and [27] guidance can be found on how to apply ANOVA for a nested experimental design. Variances were eventually combined (see [28] and [29] for further details) to obtain the precision component of uncertainty $U_{\text{precision}}$.

2.4.7. Trueness studies and evaluation of $U_{trueness}$

Trueness was investigated performing recovery studies for each substance by spiking experiments, following the experimental design and the scheme proposed in [33]. For each substance 10 tablets were grinded and the resulting powder mixed (for capsules, the content of 10 capsules was mixed) as described in Section 2.2. From this bulk, three samples containing about 100 mg of active substance were weighed and analysed to obtain a mean dosage value. Other three samples were subsequently weighed and each one was spiked with an accurately weighed amount of active substance. The amount of spiking powder was chosen so that approximately 150%, 130% and 110% of active substance could be recovered at the end of the analysis (analysis were carried out at three different concentrations).

Recovery was determined for each concentration tested as the change in observation divided by the change in concentration:

$$R = \frac{1}{n}\sum_{i=1}^{n}\frac{C_{\text{obs}(i)} - C_{\text{native}}}{C_{\text{spike}(i)}}$$

where $C_{\text{obs}(i)}$ is the observed content of each spiked sample, C_{native} is the mean content before spiking, $C_{\text{spike}(i)}$ is the amount of substance added by spiking and the number of determinations n is 3. See [22] and [33] for further details. A mean recovery was calculated from the three recovery values obtained at each concentration tested.

The uncertainty associated with the estimate of recovery U_{trueness} was calculated as reported in [33].

2.4.8. Robustness studies and evaluation of $U_{robustness}$

For each of the investigated substances the robustness testing procedure consisted in a two-level screening design as described in [38]. Seven parameters were investigated by eight determinations (each one comprised three replicates). The parameters considered were column temperature, flow rate, gradient rate, injection volume, detector wavelength, buffer pH and column type. Each parameter was examined at two levels. For instance pH was tested at 5.3 and 4.9 (5.1 is the method setting). In Table 1 the experimental design is depicted together with the chosen investigated levels.

To determine whether a variation in a parameter can affect significantly the result, a specific test was used as indicated in [32]. Although in [22] the use of a precision estimate assessed "over a short period of time" is suggested, here the time-different intermediate precision was used, following the recommendation by Hund et al. [25]. Uncertainty contributions from robustness studies were calculated as reported in [21]. Each parameter con-

Table 1
Experimental design for robustness studies

Parameters	Determination number							
	1	2	3	4	5	6	7	8
Temperature (°C)	20	20	35	35	35	35	20	20
Flow rate (ml/min)	0.8	1.2	0.8	1.2	0.8	1.2	0.8	1.2
Gradient rate (min)	26	20	20	26	26	20	20	26
Injection volume (μl)	10	30	30	10	30	10	10	30
Detection wavelength (nm)	240	220	240	220	220	240	220	240
Buffer pH	5.3	5.3	4.9	4.9	5.3	5.3	4.9	4.9
Column type	Zorbax	Zorbax	Zorbax	Zorbax	Symmetry	Symmetry	Symmetry	Symmetry

tributes with its own uncertainty. Each contribution, expressed as a relative standard deviation, was combined to obtain the global robustness uncertainty component:

$$U_{\text{robustness}} = \sqrt{\begin{array}{l} U^2_{\text{col.temp.}} + U^2_{\text{flow}} + U^2_{\text{gradient}} + U^2_{\text{inj.vol.}} \\ +U^2_{\text{wavelenght}} + U^2_{\text{pH}} + U^2_{\text{column}} \end{array}}$$

3. Results and discussion

In developing the method simplicity and affordabilty were taken into account. The extraction method was optimized to be very easy and at low cost: water was chosen as extraction solvent. The pH of the mobile phase buffer was optimised to avoid simultaneous presence of different protonated/deprotonated forms and to avoid tailing phenomena. The quite similar chromatographic retention behaviour of the analytes made gradient elution necessary to obtain complete separation of the peaks. The detection wavelength was chosen to maximise the response factor of the analytes and to minimize potential interferences from excipients. Fig. 1 shows the chromatographic separation of the six active substances. The discrimination among various antibiotics is of particular interest considering that a counterfeit drug could contain undeclared substances and often an active ingredient is substituted by a cheaper one with similar action. The method separates the six compounds permitting the detection of a fraudulent replacement of active ingredients among those considered. For other undeclared compounds different techniques have to be employed.

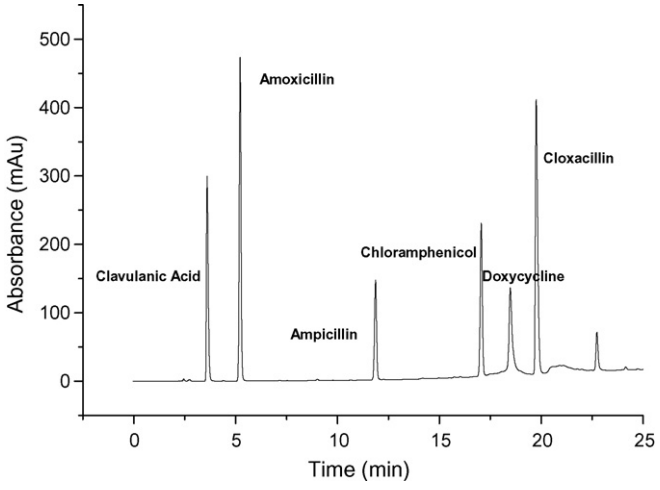

Fig. 1. Chromatographic separation of clavulanic acid, amoxicillin, ampicillin, chloramphenicol, doxycycline and cloxacillin. Chromatographic conditions are reported in Section 2.3. The chromatographic peak at about 23 min is due to the gradient jump.

Validation and measurement uncertainty estimation were designed with the aim of demonstrating the suitability and transferability of the method to control laboratories, also in developing countries. As stated in the EURACHEM Guide [19], measurement uncertainty can be estimated from data gathered during method performance determination. Barwick and Ellison [20–22] and Maroto et al. [23–24] described how to exploit validation data for uncertainty evaluation. They also propose guidelines on how to properly plan validation studies to obtain the sought information about uncertainty. Recent studies [25–39] demonstrate that uncertainty estimation from validation data handled through approaches such as the one by Barwick and Ellison can provide uncertainty estimates comparable with those obtained from inter-laboratory studies. Hence in this work validation was planned and carried out following recommendations reported in [20–24].

In Table 2 validation results and uncertainty estimates for each analyte are reported. Linearity was studied in the 10–150% range. A wide interval was chosen to verify method performance for both counterfeits (in which a rather low amount of active can be expected) and sub-standard medicines (in which an improper manufacturing may determine an excessive amount of active substance). For all analytes a good linearity was obtained.

$U_{\text{precision}}$ and repeatability are reported as relative standard deviations. Repeatability was calculated pooling the standard deviations of the six replicates performed each day by each analyst operating on each instrument. Repeatability ranged from 0.76% (amoxicillin) to 1.1% (doxycycline) while $U_{\text{precision}}$ ranged from 0.87% (amoxicillin) to 1.6% (clavulanic acid). The major imprecision in clavulanic acid determination is due to its rapid degradation. One of the two chromatographs employed in precision studies was not equipped with a refrigerated auto-sampler thus it produced lower mean results with respect to the other one. Consequently there was a considerably large *instrument-different intermediate precision*. It mostly accounts for the large precision uncertainty of clavulanic acid. Thus when no refrigerated auto-sampler is available, analyses should be conducted rapidly and samples should be kept in refrigera-

Table 2
Validation results and uncertainty estimates

	Amoxicillin	Clavulanic acid	Ampicillin	Doxycycline
Linearity	$Y = 27402x + 11$	$Y = 18404x + 33$	$Y = 9249x + 8$	$Y = 14982x - 80$
Range: 10–150%	$R^2 = 0.9998$	$R^2 = 0.9992$	$R^2 = 0.9997$	$R^2 = 0.9990$
Mean value (%)	98.8	96.9	101.8	101.4
Repeatability	0.76	0.82	0.85	1.0
Precision component of uncertainty ($U_{precision}$)	0.87	1.6	0.89	1.2
Mean Recovery	0.95	0.94	0.99	0.96
Trueness component of uncertainty ($U_{trueness}$)	2.2	2.1	2.2	2.3
Robustness				
Parameters investigated: T_{column}, flow rate, gradient rate, injection volume, detector wavelength, pH, column type	Changes in parameters do not affect the method performance	Method performance affected by changes in column temperature	Changes in parameters do not affect the method performance	Changes in parameters do not affect the method performance
Robustness component of uncertainty ($U_{robustness}$)	1.1	2.2	0.6	1.5
Combined uncertainty (U_c)	2.6	3.4	2.5	3.0

Repeatability and the various components of uncertainty are reported as percentage relative standard deviations (%RSD).

tor during the whole analytical process. Doxycycline shows a slightly larger repeatability standard deviation in comparison with the other active substances: it is correlated with some difficulties in peak integration resulting from a slight tailing. Manual integration in particular appears prone to a wider variability in comparison to the automatic process. This one, on the other hand, although more precise, may incur in blunders. Nevertheless, automatic integration is preferable in this case and should be employed whenever available.

For each substance no significant bias was detected as the mean recovery is not statistically different from 1 at each concentration tested (150%, 130% and 110%). Uncertainty values show that the method performs similarly in terms of trueness for all the investigated substances.

Robustness results for each substance are reported in Table 3, together with uncertainty components for each parameter and for the whole robustness study. In robustness determination the effect of temperature was examined at 20 °C and 35 °C (25 °C is the method setting). An asymmetric interval was chosen: it was deemed sensible to evaluate higher temperatures since the method proposed is reasonably expected to be used in tropical countries. In robustness study a wide variation range was generally considered for each parameter to account for a possible insufficient control on them during the analysis.

The method demonstrated to be robust for all the investigated parameters. Only clavulanic acid showed sensitivity toward column temperature variation. Together with precision experiments this suggests that the method performs better for this substance when column and auto-sampler are thermostated, preferably at low temperatures. Since the method is expected to be used mostly in developing countries, the large temperature range chosen for robustness investigation permitted to gain an uncertainty component that better describes a potential lack of temperature control. Substantial uncertainty contributions from temperature were consequently observed. Quite large contributions are also brought by pH and column type variation for every substance (see Table 3).

Finally the combined uncertainties obtained for the studied substances ranged from 2.5 to 3.4%. All values lay within the interval of 0.5–2.0 times the Horwitz' value [40–41] (which is 2.0% for ampicillin, 2.2% for amoxicillin, 2.3% for doxycycline, 2.9% for clavulanic acid), showing no unexpected behaviour.

The method was eventually applied to samples originating from Chad's informal market. Fig. 2 shows the chromatogram of a sample of amoxicillin from Chad (Fig. 2A) and a sample of the same antibiotic from the Italian market (Fig. 2B). The chromatogram in Fig. 2A evidences a dramatically low amount of amoxicillin in tablets from Chad (5.7% of label claim), showing their fraudulent nature. Sub-standard samples were detected

Table 3
Robustness uncertainty components for each studied parameter and total robustness uncertainty ($U_{robustness}$)

Active substance	Uncertainty contribution for each parameter							
	T	Flow rate	Gradient rate	Injection volume	Detection wavelength	Buffer pH	Column type	Total robustness uncertainty $U_{robustness}$
Amoxicillin	0.63	0.02	0.21	0.04	0.13	0.63	0.63	**1.1**
Clavulanic acid	1.90	0.02	0.24	0.04	0.14	0.71	0.71	**2.2**
Ampicillin	0.31	0.01	0.10	0.02	0.06	0.31	0.31	**0.6**
Doxycycline	0.87	0.03	0.29	0.05	0.17	0.87	0.87	**1.5**

All data are expressed as percentage relative standard deviations (%RSD). Uncertainty contributions have been conventionally rounded to two decimal places. Total robustness uncertainty has been conventionally rouded up to 1 decimal place.

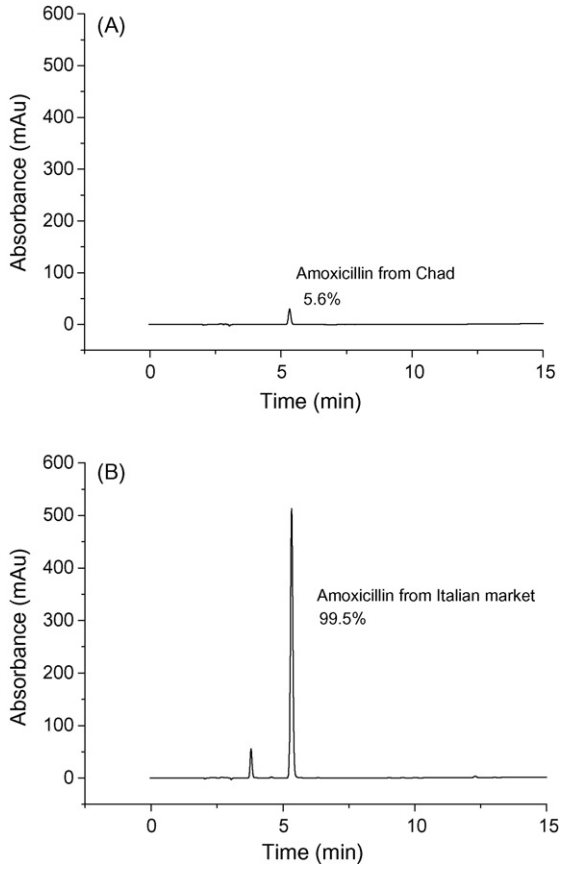

Fig. 2. Chromatographic profile of commercial amoxicillin tablets: sample purchased on informal market in Chad (A) and sample purchased on the Italian market (B). Quantitative analysis evidenced a dramatic low amount (5.6% of label claim) of amoxicillin in the sample from Chad. The chromatographic peak at about 4 min in chromatogram B is an amoxicillin related substance.

too, i.e. samples with a lower (but not dramatically) or, in some cases, a higher quantity of active substance, probably due to non compliance with Good Manufacturing Practices (data not shown).

4. Conclusions

This paper presents a new simple method for the simultaneous screening of various antibiotics in potentially counterfeit drugs. It allows to separate six of the most counterfeited antibiotics in a single chromatographic run. Validation protocol and uncertainty assessment were specifically designed envisaging a transfer of the method to quality control laboratories of developing countries. To this aim robustness was ascertained taking into account a possible lack of control on some parameters (e.g. column temperature effect). An extensive assessment of intermediate precision was accomplished to partially compensate for the lack of interlaboratory studies and to provide a more reliable estimate of uncertainty. Uncertainty estimation was used to assess the quality of data collected by this method. It also helped to have a better insight into method performance. In particular, evaluation of precision and robustness components of uncertainty evidenced the need for special care in specific situations (e.g. peak integration in doxycycline analysis, handling of clavulanic acid solutions during the course of the analysis). Finally method linearity was demonstrated in a rather wide range, to ensure method suitability for the analysis of both low dose counterfeits and sub-standards with a higher amount of active ingredient.

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