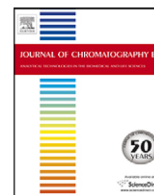
Journal of Chromatography B, 877 (2009) 2375–2379

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb



Use of the total error approach to evaluate the performance of a semi-quantitative immunological method (BIACORE method) for detecting sulfamethazine in bovine milk☆

Michel Laurentie [a,*], Valérie Gaudin [b]

[a] Pharmacokinetics-Pharmacodynamics Unit, Laboratory for studies and research on veterinary medicinal products and disinfectants, Afssa Fougères, BP 90203, La Haute Marche, 35302 Fougères, France

[b] Veterinary Drug Residues Unit, Laboratory for studies and research on veterinary medicinal products and disinfectants, Afssa Fougères, BP 90203, La Haute Marche, 35302 Fougères, France

ARTICLE INFO

Article history:
Received 22 August 2008
Accepted 23 December 2008
Available online 9 January 2009

Keywords:
Validation
Error total
Immunoassay
Sulfamethazine
Milk

ABSTRACT

A semi-quantitative immunological method (BIACORE method) for detecting sulfamethazine in bovine milk was developed and validated using the total error approach. The acceptance limits were set at ±40% and the risk of procedure of $(1-\beta)$ proportion measurements falling outside the acceptance limits was chosen at 5%. Different response functions were tested on the basis of the accuracy index (I_A). The best model was a weighted $(1/X^2)$ quadratic regression and the simplest one was an unweighted quadratic regression. This approach identified the weak point of the method, which was precision. Finally this BIACORE method was able to detect positive samples containing sulfamethazine in the dosing range between 50 and 150 ng/ml.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Sulfonamides are commonly used for prophylactic or therapeutic purposes in veterinary medicine. They are also used as coccidiostatic agents in poultry and ruminants or zoonotic molecules in cattle.

In the European Union, the Maximum Residue Limit (MRL) [1] has been established for all molecules of the sulfonamides family. This has been set at 100 μg/kg in milk and muscle [2].

Generally samples from the residue control programme are firstly analysed by a screening method in a first step and positive samples are subsequently analysed by a confirmatory method e.g. physicochemical method.

Screening methods should be qualitative methods, although not always, because in some cases an MRL has been set. A combination of residue presence or absence detection on one hand, and concentration assessment on the other hand is the applied, in relation to these regulatory limits. This is why a semi-quantitative method is generally developed, and it is necessary to validate this method with the same criteria as used in a fully quantitative method i.e. accuracy, precision, LOQ etc.

Several screening methods have been reported by Wang et al. [3] in a review including physicochemical, immunoassay and microbiological methods. Generally gas chromatography, high-performance liquid chromatography or liquid chromatography with mass detection are too expensive for screening purposes and they are preferably used to confirm the positive results. Microbiological methods are used, but they are time-consuming and the poor sensibility and lack of specificity might give false results and the results should therefore be used carefully.

Immunoassay methods are very useful for screening veterinary drugs because they are simple, rapid, sensitive and inexpensive. Furthermore, the antibodies for sulfonamides are very specific. These methods have been reported to detect one sulphonamide – sulfamethazine (SMZ) – in different matrices (milk, bile, urine and muscle) and in different species (pig, cow) [4–10].

We have developed a method based on a monoclonal antibody Mab21C7 to detect and quantify SMZ at the MRL. This BIACORE method was able to detect eight different sulfonamides in milk and muscle matrices. SMZ was chosen out of the eight sulfonamides as the reference sulfonamide, to establish the standard curves.

First of all, the validation data were analysed according to Decision 2002/657/EC [11] and were published [12].

In this paper, we decided to re-analyse the data using the accuracy profile and to study the applicability of this approach for

☆ This paper is part of a special issue entitled “Method Validation, Comparison and Transfer”, guest edited by Serge Rudaz and Philippe Hubert.

* Corresponding author. Tel.: +33 2 99 94 78 78; fax: +33 2 99 94 78 80.
E-mail address: m.laurentie@afssa.fr (M. Laurentie).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.12.069

analysing data from immunoassay validation. The re-analysis was only performed on performance criteria such as trueness, repeatability, intermediate precision and LOQ. Specificity and selectivity were not taken into account and were studied extensively in the published paper (12).

The accuracy profile is based on the total error approach that has been described in the harmonization guidelines of the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP) [13–15]. This approach has been used extensively for physicochemical methods [16] and Findlay et al. [17] recommended using it for immunoassay validation in the pharmaceutical field. We suggest investigating the usefulness with an immunoassay method for residues of veterinary drugs.

The accuracy profile is characterized by the use of two-sided β-expectation tolerance intervals calculated at each concentration level. It is necessary to define the acceptance limits and the risk of procedure $(1-\beta)$ of proportion measurements falling outside the acceptance limits. The risk was chosen at 5% and acceptance limits were set at ±40% in accordance with European Decision 2002/657/EC [11] and as described in a previous publication [16].

2. Experimental

2.1. Instrumentation

Development and validation studies for milk protocol were performed in full with the Biacore™ *X* system.

Biosensor methods measure the interactions between biological molecules without labelling and in real time. Biosensor technologies such as surface plasmon resonance (SPR) have already been used to detect antimicrobial residues in food of animal origin [4–10,18–20]. The most common SPR device is the BIACORE™ system (GE Healthcare, Sweden). This is based on the detection of an optical phenomenon. The change in refractive index of the solution close to the sensor surface is detected, which is proportional to the mass change on the sensor chip surface. In the case of antimicrobials, which are very small molecules, the antimicrobial is bound to the sensor chip surface. The antibody solution is then mixed with standards or samples. If antimicrobial residues are present in the sample, part of the antibody is bound to the antimicrobial residue. This means that less free antibodies are available to bind to the sensor chip surface and that the signal in Resonance Units (RU) would be inversely proportional to the antimicrobial concentration in the sample.

2.2. Antibodies and chemicals

The monoclonal antibody, clone 21C7, against sulfonamides was produced and kindly supplied by Fortune Kohen (Weizmann Institute of Science, Rehovoth, Israel) in lyophilized form and stored between 4 and 8 °C. The CM5 sensor chips (research grade), HBS buffer pH 7.4 as eluent (consisting of 10 mM Hepes (4-[2-hydroxyethyl]piperazine-1-ethane-sulfonic acid), 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) Surfactant P 20, and amine coupling kit containing NHS (100 mM *N*-hydroxysuccinimide in water), EDC (400 mM 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide hydrochloride in water), and ethanolamine hydrochloride, pH 8.5, were from Biacore (GE Healthcare, Sweden). SMZ and all other chemicals were from Sigma Chemical Co. (Saint-Quentin-Fallavier, France).

2.3. Immobilization of SMZ on the sensor chip surface

SMZ was covalently immobilized to the surface of one of the two channels of the sensor chip by amine coupling. The immobilization protocol has been described in a previous publication [4].

2.4. Extraction of samples

A 1 ml milk sample was transferred into a plastic tube and then centrifuged (Model GR 4.11, Jouan, Cedex, France) for 10 min at 1850 *g* at 4 °C. The fat on the top was discarded and the supernatant was diluted 20 times in the HBS buffer.

The same extraction protocol was applied to the calibration graph (spiked samples) and to unknown samples.

2.5. Sample analysis

A 50 μl volume of antibody dilution (1/40) was added to 1700 μl of HBS buffer to obtain a final antibody dilution of 1/1400. This solution could be stored for 1 month between 4 and 8 °C. The final antibody dilution (1/1400) was mixed (1:1) with the sample extract. Forty micro litres were injected at a constant flow rate of 10 μl/min. After each measurement, the surface was regenerated by injecting 5 μl of 200 mM HCl.

2.6. Calibration standards

A stock solution of sulfamethazine (SMZ) at 1 mg/ml was prepared in 0.1 M NaOH and water. From this solution, spiking solutions were prepared in the HBS buffer. A 5-point calibration graph for milk was constructed at 25, 50, 100, 200 and 300 ng/ml. A 10 μl volume of each spiking solution was added to 990 μl blank milk.

Eight series of analysis were performed and calibration samples were analysed twice.

2.7. Validation standards

From the same stock solution of SMZ, spiking solutions were prepared in the HBS buffer. Validation standards were prepared at 1/2 MRL, MRL and 1.5 MRL (50, 100 and 150 ng/ml) as stipulated in Decision 2002/657/EC [11].

For validation samples, 5 series were performed and analysed 6 times.

2.8. Validation analysis

The validation data were processed using e.noval software, version 2.0, and Seelva Version 1.0 beta 8 for logistic function (Arlenda, Liège, Belgium).

3. Results and discussion

3.1. Response function

To fit the response to concentrations, different models of regression were tested: linear, weighted linear, quadratic, weighted quadratic regression, logistic functions with 4 or 5 parameters and weighted logistic functions.

The accuracy profile depends on the response function. To select the best accuracy profile, some desirability indexes based on major validation criteria were defined [21]. The notes under Table 1 summarize the different indexes used for dosing range, trueness and precision i.e. I_{DR}, I_T, I_P. These three indexes were combined for the accuracy index (I_A).

We have retained the accuracy profile and response function associated with $I_A > 0.7$. the notes under Table 1 summarize the different indexes obtained for all function responses tested. Calculation was not possible for three models (weighted five parameter pogistic regression, weighted linear regression, and linear regression). The I_A shows that six models have an index ranging from 0.84 to 0.89. For these models, the dosing range index is equal to 1 i.e.

Table 1
Indexes of the different regression models tested, sorted by accuracy index.

Model	I_A[a]	I_{DR}[a]	I_P[a]	I_T[a]	
Weighted ($1/X^2$) quadratic regression	0.89	1.00	0.74	0.95	Fig. 1a
Weighted ($1/X$) quadratic regression	0.88	1.00	0.71	0.97	Fig. 1b
Unweighted quadratic regression	0.87	1.00	0.67	0.96	Fig. 1c
Weighted four parameter logistic regression	0.85	1.00	0.62	0.98	Fig. 1d
Unweighted five parameter logistic regression	0.84	1.00	0.61	0.98	Fig. 1e
Unweighted four parameter logistic regression	0.84	1.00	0.60	0.98	Fig. 1f
Weighted five parameter logistic regression	NC	NC	NC	NC	
Weighted linear regression	NC	NC	NC	NC	
Linear regression	NC	NC	NC	NC	

All indexes range from 0 to 1; (a) see publication 21 for formulas of the indexes; the accuracy index (I_A) is a global indicator of method performance depending on I_{DR}, I_T, I_P. The dosing range index (I_{DR}) indicates the fraction of range that is valid; when I_{DR} = 1, the whole studied range is accepted. I_T: trueness index: an index close to 1 implies that the method is almost not biased; I_P: precision index: an index close to 1 indicates that the method has a strong precision; NC: not calculated.

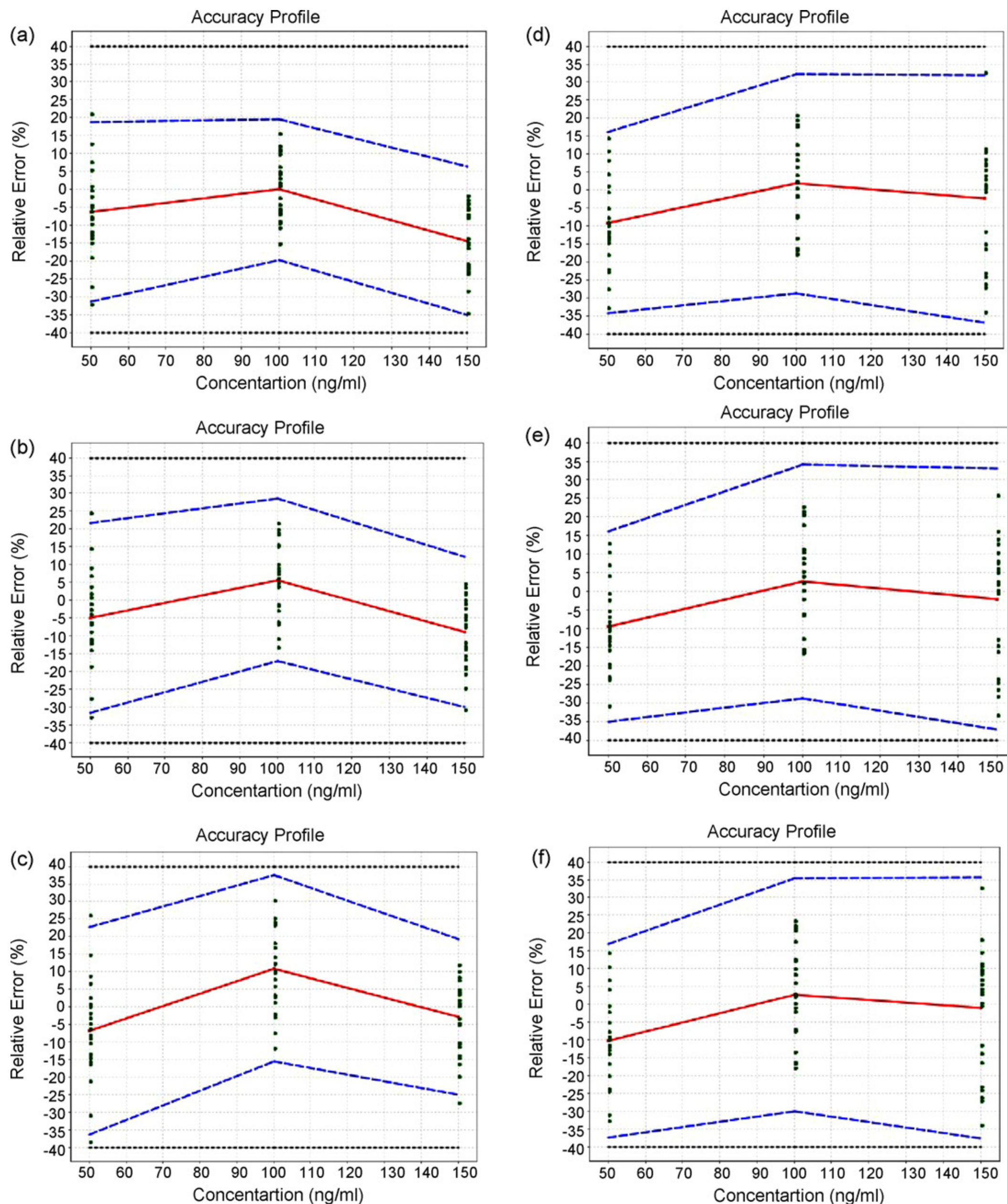


Fig. 1. Accuracy profile obtained for the measurement of the level of sulfamethazine in milk with (a) weighted ($1/X^2$) quadratic regression, (b) weighted ($1/X$) quadratic regression, (c) unweighted quadratic regression, (d) weighted four parameter logistic regression, (e) unweighted five parameter logistic regression, (f) unweighted four parameter logistic regression. Plain lines are the relative bias, the dashes lines are the β-expectations tolerance limits, the dotted curves represent the acceptance limits, the dots represent the relative back-calculated concentrations of the validation standards.

Table 2
Trueness (relative bias %), precision (RSD of repeatability and intermediate precision) obtained for models selected at MRL level.

Models	Concentration level 100 ng/ml		
	Trueness (relative bias %)	Repeatability (RSD %)	Intermediate precision (RSD %)
Weighted ($1/X^2$) quadratic regression	−0.1	6.6	8.9
Weighted ($1/X$) quadratic regression	5.6	7.0	10.2
Unweighted quadratic regression	11.0	7.8	11.7
Weighted four parameter logistic regression	1.8	8.0	13.3
Unweighted five parameter logistic regression	2.7	8.4	13.8
Unweighted four parameter logistic regression	2.6	8.1	14.2

Table 3
Comparison of validation parameters obtained by conventional or total error approach.

Parameters	MRL Level (100 ng/ml)		
	Approach		
	Total error		Conventional
	Weighted ($1/X^2$) quadratic[a]	Unweighted five parameter logistic[a]	Unweighted five parameter logistic[a]
Trueness (relative bias %)	−0.05	2.7	3.0
Repeatability (RSD, %)	6.6	8.4	8.4
Intermediate precision (RSD, %)	8.9	13.8	13.0

[a] Regression type.

the whole dosing range was allowed. Therefore, the target concentration (MRL) is included in the dosing range. The method is fit for purpose.

3.2. Trueness

If we look at the I_T index (Table 1), some discrepancies are observed between models. Generally, trueness is good with an index higher than 0.95, and it is very high for logistic models. The relative bias (%) was reported for the different models in Table 2. Generally it was low, except for weighted ($1/X$) quadratic regression or unweighted quadratic regression where values ranged from 5.6 to 11.0, respectively. However biases are acceptable for the MRL level.

3.3. Precision

In contrast, quadratic models are more precise than logistic models. In Table 1, the best value was obtained for a weighted ($1/X^2$) quadratic regression (0.74) and decreased rapidly to a low value (0.60) for a traditional four parameter logistic regression. This indicates that precision is the weak point of the method. If we look at Table 2, particularly repeatability (intra series) and intermediate precision (inter series), we see that the intermediate precision is the main factor responsible for the variability. We thus diagnosed the origin of the problem in our study.

3.4. Accuracy

In Fig. 1, accuracy profiles for the six different response functions retained were reported. Visually, considerable variability was observed for logistic regression. This was confirmed when looking at the accuracy index (I_A). Based on this index, the best model is a weighted ($1/X^2$) quadratic regression model (Table 1).

However, based on the principle of parsimony or on the FDA document [22], the simplest models such as unweighted quadratic function or unweighted four parameter logistic regression are possible.

The choice is relevant for the routine because the easiest model offers the possibility of avoiding fastidious calculations.

3.5. Limit of quantification (LOQ)

For all models tested, the whole dosing range was validated (Table 1) as shown by the dosing range index (I_{DR}). Consequently, the lower LOQ was set at 50 ng/ml and the upper LOQ at 150 ng/ml with a defined and acceptable accuracy.

3.6. Comparison of results between conventional and total error approaches.

The total error approach determines trueness and precision.Validation of SMZ analysis with the Biacore system was performed earlier with the conventional approach (12) with an unweighted five parameter logistic regression because this calculation method was directly available with the supplied software (BiaEval®, GE Healthcare, Sweden) for calculating the back concentrations. As shown in Table 3, the performance estimates obtained with the total error approach are of the same magnitude for the same model. Graph inspection (Fig. 1a) shows that by extrapolation the measuring range can be extended from 15 to 170 ng/ml. These values are only indicators and cannot be used for quantification.

Finally, the total error approach determines the accuracy of results in a global approach when the conventional approach is based on sequential analysis for accuracy, trueness or precision.

Four and five parameter logistic regression models are currently used for immunological tests. However, the total error approach showed that they are not always the best models for the response function.

4. Conclusion

The accuracy profile has been successfully applied to validate an immunoassay for the detection of sulfamethazine in milk. This approach is in accordance with decision 2002/657/EC [11] and offers the guarantee that at least 95% of the future results (unknown samples) obtained with the validated method will be within the ±40% acceptance limits.

We have tested some different response functions. It is possible to retain the best model in terms of accuracy index (I_A) (weighted ($1/X^2$) quadratic regression) or to use the simplest model (unweighted quadratic regression).

Furthermore, this approach is a tool for identifying the weak point of the method, which in this case was the precision of the method. Intermediate precision was the main factor responsible for the variability. Finally, this approach was used to validate semi-quantitative screening methods for the detection of veterinary drugs in foods. The total error approach confirmed that the developed BIACORE method was fit for purpose (screening of sulfamethazine in milk samples with a BIA assay at the target level (MRL)).

References

[1] Council Regulation (EEC) N° 2377/90, Off. J. Eur. Commun. L224 (1990) 1.
[2] EEC Regulations, N° 675/92 Off. J. Eur. Commun. L73, (1992) 8.
[3] S. Wang, H.Y. Zhang, L. Wang, Z.J. Duan, I. Kennedy, Food Addit. Contam. 23 (2006) 362.
[4] V. Gaudin, M.L. Pavy, J. AOAC Int. 82 (1999) 1316.
[5] C. Bergstrom, A. Sternesjo, P. Bjurling, S. Lofas, Food Agric. Immunol. 11 (1999) 329.
[6] S.R.H. Crooks, G.A. Baxter, M.C. O'Connor, C.T. Elliott, Analyst 123 (1998) 2755.
[7] G.A. Baxter, J.P. Ferguson, M.C. O'Connor, C.T. Elliott, J. Agric. Food. Chem. 49 (2001) 3204.
[8] C.T. Elliott, G.A. Baxter, S.R.H. Crooks, W.J. McCaughey, Food Agric. Immunol. 11 (1999) 19.
[9] T.L. Fodey, S.R.H. Crooks, C.T. Elliott, W.J. McCaughey, Analyst 122 (1997) 165.
[10] P. Bjurling, G.A. Baxter, M. Caselunghe, M. Jonhson, M.C. O'Connor, B. Person, C.T. Elliott, Analyst 125 (2000) 1771.
[11] Commission Decision 2002/657/EC, Off; J. Eur. Commun. L221 (2002) 8.
[12] V. Gaudin, C. Hédou, P. Sanders, J. AOAC Int. 90 (2007) 1706.
[13] Ph. Hubert, P. Chiap, J. Crommen, B. Boulanger, E. Chapuzet, N. Mercier, S. Bervoas-Martin, P. Chevalier, D. Grandjean, P. Lagorce, M. Lallier, M.C. Laparra, M. Laurentie, J.C. Nivet, Anal. Chim. Acta 391 (2) (1999) 135.
[14] Ph. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, STP Pharma 13 (2003) 101.
[15] Ph. Hubert, J.J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewé, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, J. Pharm. Biomed. Anal. 36 (3) (2004) 57.
[16] E. Chéneau, J. Henri, Y. Pirotais, J.P. Abjena, B. Roudaut, P. Sanders, M. Laurentie, J. Chrom. B 850 (2007) 15.
[17] J.W.A. Findlay, W.C. Smith, J.W. Lee, G.D. Nordblom, I. Das, B.S. DeSilva, M.N. Khan, R.R. Bowsher, J. Pharm. Biomed. Anal. 21 (2000) 1249.
[18] V. Gaudin, P. Maris, Food Agric. Immunol. 13 (2001) 77.
[19] V. Gaudin, J. Fontaine, P. Maris, Anal. Chim. Acta 436 (2001) 191.
[20] A.C. Huet, C. Charlier, G. Singh, S. Benrejeb Godefroy, J. Leivo, M. Vehniäinen, M.W.F. Nielen, S. Weigel, Ph. Delahaut, Anal. Chim. Acta 623 (2008) 195.
[21] E. Rozet, V. Wascotte, N. Lecouturier, V. Préat, W. Dewé, B. Boulanger, Ph. Hubert, Anal. Chim. Acta 591 (2007) 239.
[22] Guidance for industry: Bionalytical Method Validation, US Departement of Health and Human services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Biologics Evaluation and Research (CBER), Rockville, May 2001.