Journal of Food Protection, Vol. 59, No. 11, 1996, Pages 1223–1226

Comparison of Biosensor, Microbiological, Immunochemical, and Physical Methods for Detection of Sulfamethazine Residues in Raw Milk

CATARINA MELLGREN,[1]* ÅSE STERNESJÖ,[1] PHILIPP HAMMER,[2] GERTRAUD SUHREN,[2] LENNART BJÖRCK,[1] and WALTER HEESCHEN[2]

[1]*Department of Food Science, P. O. Box 7051, Swedish University of Agricultural Sciences, S-750 07 Uppsala, Sweden; and* [2]*Institute for Hygiene, Federal Dairy Research Centre, P. O. Box 6069, D-241 21 Kiel, Germany*

(MS# 95-279: Received 30 October 1995/Accepted 4 March 1996)

ABSTRACT

A biosensor assay based on biospecific interaction analysis (BIA) was compared with already existing methods for detection of sulphamethazine (SMZ) residues in milk. Microbial inhibitor and receptor assays, an enzyme-linked immunosorbent assay (ELISA), high-pressure liquid chromatography (HPLC), and BIA were used to analyze milk samples from SMZ-treated cows. The results of the commercially available tests (Delvotest SP Special, BR-test Blue Star, Charm II test) were in agreement with the claimed sensitivity of the respective assays. The agreement between the quantitative methods (ELISA, HPLC, BIA) varied. The microbial inhibitor assays and BIA were also used to screen 330 tanker milk samples. All samples were negative in the inhibitor tests, whereas the BIA indicated the occurrence of less than 0.9 μg of SMZ per kg of milk in 5 samples and 1.5 ± 0.6 μg/kg in one sample. HPLC indicated the presence of SMZ in the latter sample, although the concentration was below the detection limit of the method. The advantages offered by the BIA: no sample preparation, high sensitivity, and rapid, fully automated analysis in real time make the technology an interesting alternative to existing screening methods within future food-quality control systems.

Key words: Comparison, methods, detection, sulfamethazine, cow milk, raw milk

The widespread use of antimicrobial agents to improve animal performance and control disease has become an issue of considerable importance in animal husbandry. The problems associated with the occurrence of residues in foods of animal origin have led to reassessment of the use of antimicrobial drugs *(7)*. Higher standards of food-safety assurance are being required by society *(16)*, and maximum residue levels (MRLs) based on toxicological evaluations have been established for a number of substances *(3)*. In many cases, traditional microbial screening methods have insufficient sensitivities to meet these new regulations *(14)*. Routine monitoring of residues and contaminants in food by use of classical physiochemical techniques, e.g., chromatographic methods and mass spectrometry, is often precluded due to the level of experience, skills, and costs required for the analysis. Moreover, these methods are often tedious and may require elaborate sample clean-up procedures, which limit the sample throughput. In this regard, the use of immunoassays has become increasingly popular for monitoring levels of therapeutic substances *(4)*. The high specificity and sensitivity of immunological techniques has led to the development of a variety of rapid screening methods for detection of toxic and undesirable components in food, while still reducing the use of expensive equipment and test time. Also these methods must, however, be improved to meet a growing demand for continuous quality control and safety monitoring with on-line instrumentation.

Advances in both biotechnology and electronics have accelerated the development of biosensors *(5)*. A biosensor may generally be defined as an analytical device composed of two elements: a biological element that detects the analyte and a physical transducer which converts this detection event into an electronic signal. Immunosensors are essentially biosensors that use antibodies as detector molecules. Based on the mode of signal transduction, almost all biosensors fall into four categories, i.e., optical, mass, electrical, and thermal biosensors. Although the technology is still in its infancy, the potential of optical biosensors in the control of contaminants and residues in meat and dairy products is believed to be considerable. A recently developed, competitive assay for the detection of sulfamethazine *(12)* was the first application of biospecific interaction analysis (BIA) and the BIAcore™ system (Pharmacia Biosensor, Uppsala, Sweden) for determination of antibiotic residues in milk. In this paper, the assay is compared with prevailing methods, i.e., microbial inhibitor and receptor assays, an enzyme-linked immunosorbent assay (ELISA), and high-pressure liquid chromatography (HPLC).

* Author for correspondence. Tel: +46-18-672040; Fax: +46-18-672995; E-mail: Catarina.Mellgren@lmv.slu.se

MATERIALS AND METHODS

Experimental layout

The present investigation was a collaborative study between the Institute of Hygiene, Federal Dairy Research Centre, Kiel, Germany and the Department of Food Science, Swedish University of Agricultural Sciences, Uppsala, Sweden. All milk samples except the controls were collected at the Institute of Hygiene. All analyses except the BIA, which was conducted at the Department of Food Science, were performed at the Institute of Hygiene. For preparation of standard samples with known concentrations of sulfamethazine (SMZ), antibiotic-free milk from the dairy research farms of the University and the Federal Dairy Research Centre was used.

Milk samples

Milk from SMZ-treated cows. SMZ was intravenously administered to a total of 4 healthy cows on two different occasions at the Federal Dairy Research Centre research farm. The dosage was 0.2 ml of Selectavet Sulfadimidine™ (Alvetra, Neumuenster, Germany) (33%, wt/vol)) per kg of body weight. In the first experiment, milk samples (A) from an individual cow were collected. In the follow-up experiment, milk samples from 3 cows were collected and commingled to one pooled sample (B) at each milking. The milk samples were collected from the first day of the treatment and during 10 (A) or 6 (B) successive days in connection with ordinary morning and evening milking. The samples were split into several subsamples and then stored frozen (A) or freeze-dried (B) until analysis. One subsample was sent to the Department of Food Science for the BIA.

Tanker milk samples. Tanker milk samples (330) were randomly collected during 4 months from an ongoing survey for residues of antimicrobials within an integrated detection system in Schleswig-Holstein *(8).* The samples were analyzed with two microbial inhibitor assays (BR-test Blue Star and Delvotest SP Special) and were then frozen and sent to the Department of Food Science, where they were subjected to BIA. Tanker milk samples found positive for SMZ in the BIA were refrozen and returned to Kiel, where they were analyzed by using HPLC.

Methods

Microbial inhibitor assays. Two microbial inhibitor assays were used to analyze milk samples from the SMZ-treated cows and the tanker milk samples, the Delvotest SP Special (Gist-Brocades, Delft, the Netherlands) and the BR-test Blue Star (Laboratorium Enterotox, Krefeld, Germany). The methods were conducted according to the manufacturers' instructions. The sensitivity to SMZ was 100 to 200 µg/kg of milk for both tests.

The microbial receptor assay. The Charm II microbial receptor assay (Charm Sciences Inc., Malden, MA, USA) for sulfonamides was used to analyze milk samples from the SMZ-treated cows. The method was conducted according to the manufacturer with the modifications previously described *(10).* The sensitivity of the assay was approximately 10 µg of SMZ per kg of milk.

ELISA: Polyclonal anti-SMZ antibodies. Rabbits were immunized with a conjugate between bovine serum albumin and SMZ. The preparation of the immunogen and the immunization of rabbits have been described elsewhere *(6).* Polyclonal antibodies were precipitated from the serum with ammonium sulfate *(9)* and were used in both the ELISA and the BIA.

Method. The milk samples from the treated cows were subjected to a previously described competitive ELISA for sulfonamides *(6).* The detection limit of the assay was approximately 10 µg of SMZ per kg of milk.

BIA. BIA was used to assay milk samples from both tankers and the SMZ-treated cows, and the analysis was carried out as previously described by Sternesjö et al. *(12).* The repeatability of the method was 1.9% (n = 10) and the variation between SMZ-free herd milk samples (n = 39) was 2.4%. In the present study, the assay had a detection limit of 0.9 µg of SMZ per kg of milk. The response of an unknown sample was compared with a standard curve (1 to 10 µg/kg) constructed by least-squares linear regression on the logarithms of the drug concentration versus the response.

HPLC. All milk samples from the SMZ-treated cows were analyzed by HPLC as described by Suhren and Heeschen *(15).* In addition, HPLC was used to analyze for SMZ in tanker milk samples found positive by the BIA. The limit of detection and the limit of quantification of the method was 3 and 10 µg of SMZ per kg of milk, respectively. Preliminary data (n = 4 laboratories) showed that the repeatability and the reproducibility of the method at the 10 µg/kg level were 5.9 and 7.9%, respectively. SMZ was quantified by comparison with a standard curve constructed by least-squares linear regression using sulfamethizole as the internal standard. SMZ was identified by addition of a sulfonamide standard solution to the extracted samples and the samples were re-analyzed.

RESULTS

Analysis of milk samples from SMZ-treated cows

SMZ was injected on day 1 after morning milking and the results in the different assays for 10 consecutive milkings are shown in Table 1. Both samples A and B showed positive results in the two microbial inhibitor assays, Delvotest SP Special and BR-test Blue Star, on days 1 and 2. On day 3, the morning milk samples showed questionable results in both assays. The quantitative methods indicated SMZ concentrations that were close to the detection limits of the inhibitor assays (100 to 200 µg of SMZ per kg of milk) in both A and B.

The Charm II microbial receptor assay showed positive results for both A and B throughout day 4. HPLC analysis indicated the presence of less than 10 µg of SMZ per kg of milk in both A and B morning milk samples of day 4, which was the approximate detection limit of the Charm II.

With a few exceptions, the results in both the BIA and the ELISA showed good agreement with the HPLC results in the lower concentration interval, i.e., 1 to 100 µg/kg (Figure 1). At higher concentrations, however, the agreement was

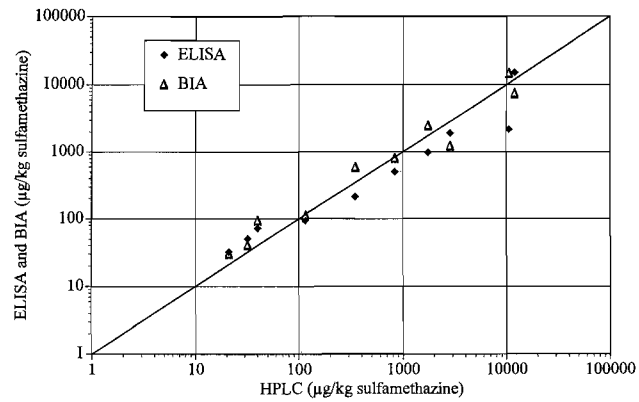

FIGURE 1. *Agreement between results from BIA and ELISA, respectively, and HPLC in assays for SMZ residues of milk samples from SMZ-treated cows.*

TABLE 1. *Assays by various methods for sulfamethazine (SMZ) residues in milk from treated cows*

	Sulfamethazine residue											
	Present (+) Absent (−)						μg of SMZ per kg of milk					
	Delvotest SP Special (100–200)[a]		BR-test BlueStar (100–200)		CharmII (10)		ELISA (10)		HPLC (10)		BIA (0.9)	
Milking (day:time)[b]	A[c]	B	A	B	A	B	A	B	A	B	A	B
1:M	−	−	−	−	− (93)[d]	− (93)	0	6	0	0	0	0
1:E	+	+	+	+	+ (16)	+ (15)	2,170	15,000	10,500	11,960	14,706	7,400
2:M	+	+	+	+	+ (15)	+ (18)	980	1,900	1,750	2,847	2,470	1,220
2:E	+	+	+	+	+ (23)	+ (18)	213	500	348	832	588	800
3:M	+/−	+/−	+/−	+/−	+ (23)	+ (18)	72	93	40	116	94	110
3:E	−	−	−	−	+ (23)	+ (23)	32	50	21	32	30	40
4:M	−	−	−	−	+ (31)	+ (38)	20	25	[9][e]	[9]	16	9
4:E	−	−	−	−	+ (36)	+ (29)	14	[8]	[8]	[4]	11	3
5:M	−	−	−	−	− (65)	− (56)	15	[4]	[4]	[1]	6	3
5:E	−	−	−	−	− (68)	− (63)	11	[1]	[3]	[1]	3	2

[a] Assay sensitivity, μg of SMZ per kg of milk.
[b] M, morning; E, evening. Cows injected after day 1 morning milking.
[c] A, milk from 1 cow; B, pooled milk from 3 cows.
[d] Values within parentheses are expressed in % of a negative control milk sample, 10 μg/kg corresponding to a ratio of 45%.
[e] Values within square brackets are below the quantification level of the method.

reduced. When the results from the BIA and the ELISA were compared, generally more divergences were found.

Analysis of tanker milk samples

Altogether 330 tanker milk samples, all of them negative in the microbial inhibitor assays, were assayed for SMZ residues by using the BIA. Five of the samples were found to contain concentrations below 0.9 μg of SMZ per kg of milk. In addition, one sample contained 1.5 ± 0.6 μg of SMZ per kg. In the analysis by HPLC, this sample showed a distinct peak at the retention time for SMZ. The concentration was, however, below the quantification limit (10 μg/kg) of the HPLC method.

DISCUSSION

Sulfonamides are a family of chemotherapeutics that are widely used for therapeutic and prophylactic purposes in animal diseases. In the treatment of mastitis, sulfonamides are usually administered in the case of infections caused by gram-negative pathogens, e.g., *Escherichia coli*. Toxicological data show that sulfonamides have antithyroid effects in both animals and humans *(17)*. Considering the health aspects of the consumer, a maximum residue limit (MRL) of 100 μg/kg has been established for the sum of sulfonamides in milk in the EU *(2)*. In comparison, the Codex MRL is 25 μg/kg *(17)* and the US safe level is 10 μg/kg *(1)* for SMZ.

The use of microbial inhibitor assays in screening for antimicrobial residues in milk is often criticized for being too insensitive to sulfa drugs. The present study shows that both the Delvotest SP Special and the BR-test Blue Star only detected the samples that contained the highest concentrations of SMZ, i.e., samples collected before completion of the 3 days withdrawal time. Because the MRL is close to the detection limits of both the Delvotest and the BR-test Blue Star, it is uncertain whether the tests would detect SMZ at the regulatory level.

The Charm II microbial receptor test detects the family of sulfonamides at comparatively low concentrations. Depending on the composition and quality of the milk sample, however, interferences by various milk components occasionally make it difficult to evaluate the results *(13)*. In the present study, the test indicated the presence of sulfonamides during the first 4 days. In the analyses by the quantitative methods, the SMZ concentrations in the samples from day 4 were found to be below or close to 10 μg/kg, which was in agreement with the predetermined sensitivity of the Charm II.

Both the ELISA and the BIA generally showed good agreement with the HPLC, although the results were biased. One possible reason for this observation may be the sample dilution procedure, which may influence the results to a varying degree in the different methods. In the case of the BIA, the linear part of the standard curve is rather narrow (1 to 10 μg/kg). This means that the risk for dilution errors increases with increasing concentrations. It is also possible that there are effects which are related to the dilution of the matrix; this has to be further investigated.

The BIA has hitherto been used to study macromolecular interactions in a wide range of application areas, ranging from basic research to drug design, monoclonal antibody production, and infectious disease research *(11)*. To our knowledge, this study is the first application of a BIA for routine determination of veterinary drug residues in milk or in any other animal-derived food. There are several advantages of the BIA compared with already existing techniques used in the determination of antibiotic residues in milk. Assaying SMZ by using BIA requires no sample preparation; it is fully automated and the time required for analysis

is short, approximately 20 min including regeneration of the surface between samples. Moreover, the method is very sensitive and specific, allowing quantification of concentrations in the low microgram per kilogram level. At present, BIA assays for other antimicrobial substances are being developed, with the intention of enabling simultaneous determination of four different substances in one sample within less than 10 min. We believe that the BIA offers sufficient advantages to be an interesting alternative within an integrated system for the future control of residues and contaminants in food.

ACKNOWLEDGMENTS

The authors thank Pharmacia Biosensor, Uppsala, Sweden, for their support and technical assistance in Sweden. The Swedish Dairies Association is gratefully acknowledged for partly financing the project. In Germany, the project was supported by a research grant of the EEC (Contract no. 1116/92-2.1).

REFERENCES

1. Code of Federal Regulations, Title 21. 1991. Food and drugs. Office of the Federal Register, U.S. Government Printing Office, Washington, D.C.
2. EEC 1992. Commission regulation no. 675/92 amending annexes I and III of Council Regulation (EEC) no. 2377/90 of March 18 1992 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Off. J. E. C. 73:8–14.
3. EEC. 1990. Council regulation no. 2377/90 of June 26 1990 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Off. J. E. C. 224:1–8.
4. Deshpande, S. S. 1994. Immunodiagnostics in agricultural, food, and environmental quality control. Food Technol. 48(6):136–141.
5. Deshpande, S. S., and R. M. Rocco. 1994. Biosensors and their potential use in food quality control. Food Technol. 48(6):146–150.
6. Grether, A., P. Hammer, and W. Heeschen. 1994. Zum immunchemischen Nachweis ausgewählter Sulfonamide in Milch. Kieler Milchwirts. Forsch. 46:101–127.
7. Hady, P. J., J. W. Lloyd, and J. B. Kaneene. 1993. Antibacterial use in lactating dairy cattle. J. Am. Vet. Med. Assoc. 203:210–220.
8. Heeschen, W., and G. Suhren. 1993. Antibiotics and sulfonamides in milk: significance, evaluation, maximum residue limits (MRLs) and concepts of detection from an international point of view. Kieler Milchwirts. Forsch. 45:43–60.
9. Hebert, G. A., P. L. Pelham, and B. Pittman. 1973. Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse and goat antiserum. Appl. Microbiol. 25:26–36.
10. International Dairy Federation. 1991. Detection and confirmation of inhibitors in milk and milk products. Bull. Int. Dairy Fed. 258:73–76.
11. Milett, R. 1993. Foreword. *In* Report from the third European BIA symposium, 23–24 September 1993, London. Pharmacia Biosensor, Uppsala, Sweden.
12. Sternesjö, Å., C. Mellgren, and L. Björck. 1995. Determination of sulfamethazine residues in milk by a surface plasmon resonance-based biosensor assay. Anal. Biochem. 226:175–181.
13. Suhren, G. 1993. Experiences with the Charm II test (Kiel modification) for the detection of antibiotics in milk for consumption. Bull. Int. Dairy Fed. 283:47–52.
14. Suhren, G., P. Hammer, and W. Heeschen. 1994. Hemmstoffe, Antibiotika und Sulfonamide. Kieler Milchwirts. Forsch. 46:237–248.
15. Suhren, G., and W. Heeschen. 1993. Detection of eight sulfonamides and dapsone in milk by a liquid chromatographic method. Anal. Chim. Acta 275:329–333.
16. van Dresser, W. R., and J. R. Wilcke. 1989. Drug residues in food animals. J. Am. Vet. Med. Assoc. 194:1700–1710.
17. World Health Organisation. 1989. Evaluation of certain veterinary drug residues in food. Thirty-fourth report of the joint FAO/WHO expert committee on food additives. World Health Organisation technical report Ser. 788, 32–40.