A COMPARISON OF TLC, GC-MS, HPLC AND ELISA-METHODS FOR SULFAMETHAZINE DETERMINATION IN PORK

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INTRODUCTION

Sulfamethazine is widely used for treatment and prevention of bacterial infections in swine. In 1987 and 1988 the American authorities started worrying about the possibility that sulfamethazine could be a carcinogen. As a result, a very effective system for controlling residues in food for human consumption was called for. Denmark at present tolerates 0,10 ppm as the maximum concentration in meat. A control plan for the meat industry then had to meet the following demands: It must be able to accurately quantify at least 0,06-0,07 ppm in meat. It must be very precise and, in addition, fast and cheap.

According to Randecker et al. (1) the blood contains approximately 4 times as much sulfamethazine as the muscles. This means that an analysis on blood should at least be able to quantify 0,20 ppm.

The purpose of this study was to investigate the possibility of using a rapid immunological technique on blood serum for primary screening, and a TLC procedure (3) for confirmation of possible violative muscles. The TLC-method was chosen because it is one of the methods used by the USDA Food Safety and Inspection Service, and because of its ability to analyse many samples simultaneously, while maintaining selectivity and sensitivity through flourescence detection. In addition, the HPLC procedure was used for control of the immunological technique, and a GC-MS (2) procedure for control of the TLC-procedure. The study also evaluates the corresponding factor between blood and muscle-tissue concentrations.

MATERIALS AND METHODS

A. ELISA-test on blood serum

Fresh blood, collected at the slaughter house, was cooled to approximately 5°C and sent to the laboratory. The samples were then centrifuged for 10 min. at 3000 rpm and the blood serum collected. If the samples were not analysed within 24 hours they were stored at -20°C. The analyses were performed using a commercial competetive ELISA-kit from Idetek Inc. (California). The kit can detect 0,1 ppm or less. All samples which after the first test showed concentrations of 0,20 ppm or more were analysed twice. A Titertek Multistepper was used for all operations, and the plate was read by a Titertek Multiscan Plus Mk. II operating at 405 nm. Calculations were made by a programme kindly provided by J. Flink, NOVO Food Diagnostics, who also assisted in data processing. It uses a linear standard curve.

B. HPLC on blood serum

Blood serum was prepared as for the ELISA-test. Any remaining proteins were separated by shaking the blood serum with 5% trichloracetic acid. The supernatant was injected directly into the HPLC system.

The equipment used was M-510 pumping device, WISP 710B automatic sample injector and M-81 UV detector from Waters.

Data were obtained and integrated by Waters Maxima 820 software programme.

The chromatographic conditions were: column Nucleosil, Reverse phase C8, 3µm, operating at 25°C; eluent acetic buffer pH 4,9: acetonitrile 75:25

and flow 0,6 ml/min. Detection was 272nm. The detection limit was 0.05 ppm and the recovery was 100%.

C. TLC on muscles

The analyses were performed as described in reference (3). Muscles were collected at the slaughterhouse, frozen and sent to the laboratory. They were kept frozen at -20°C until the analyses were carried out. The principle in the analytical procedure is that the samples are spiked with a similar compound - sulfapyridine - in a known amount. The idea is that losses during analysis are almost similar for sulfapyridine and sulfamethazine, so calculations are made on the sulfamethazine: sulfapyridine ratio.

After several extractionsteps, harnessing the sulfacompound's pH-dependent polarities, the concentrated extracts are spotted on TLC-plates with channels and preabsorbent zones. The plates are developed, dried and then dipped, in a flourescamine solution. The flourescamine reacts with primary amines to form a flourescent product. The plate is evaluated in a densitometer. The sulfamethazine contents are calculated using a standard curve with the ratio plotted against the concentration. The method can quantify 0.06-0.07 ppm, but can detect less. All samples with calculated contents of 0,05 ppm or more have been analysed twice.

The densitometer used is a Camag TLC Scanner II operated with 410 nm excitation wavelength, and with a 460 nm cut-off filter for emmision readings. The densitometer is connected to an integrator (Merck-Hitachi 000).

D. GC-MS on Muscles

The analyses are performed as described in reference (2). The principle is that the samples are spiked with ^{13}C-sulfamethazine (KOR Isotopes, Cambridge) in a known amount. Then, they are extracted and concentrated. Subsequently, the extracted sulfamethazine reacts with diazomethane to form N^1-methyl sulfamethazine. After another concentration step the samples are injected into the GC-MS-system. The analyses were conducted on a VG-TS250 Trisector instrument operated in selected ion monitoring under the following conditions: Electron energy 70 eV, photomultiplier 550 V, source temperature 140°C, integration time 50 ms/mass monitored.

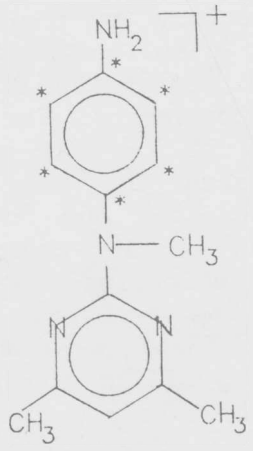
A 0.22 mm i.d. x 25 m, fused silica BP-5 capillary column with film thickness of 0.25 µm was used to affect separation.

GC conditions were as follows: Injection point temperature 280°C, column temperature 150°C for 1 min., and then increased with 8°C/min. to 280°C and held for 5 min. Column head pressure 100 KPa.

The column is interfaced directly to the ion source.

The detection limit is 15 - 25 ppb dependent on the amount of meat extracted.

Calculations were made using the 227/233 ion mass ratio (see Fig. 1) and a linear standard curve with the ion mass ratio plotted against the amount of ^{12}C-sulfamethazine.



The * indicate the positions for ^{13}C

Figure 1.

RESULTS AND DISCUSSION

Table 1 shows the results from the methods used for muscle analysis. Table 2 shows the results for the blood analysis. Not all pigs were analysed by all methods because of too small samples or difficulties in getting blood samples. The tables show the individual results, the average and the standard deviation between the GC-MS method and the TLC method, and between the HPLC method and the ELISA-test.

Table 1 shows that the methods for muscle analysis yield comparable results, with the exception of sample no 13. It shows results which are clearly unacceptable; the reason for this is now being examined. An error was probably made during the extraction for the GC-MS procedure, as the blood from this pig also shows a high sulfamethazine content.

The standard deviation varies with the sulfamethazine concentration and as expected we see the maximum near the detection limits of the methods.

The TLC method tends to show slightly higher sulfamethazine contents than does the GC-MS method. This can be explained by the fact that the methods use different internal standards. The added ^{13}C-sulfamethazine in the GC-MS procedure will give the same recovery as the naturally occuring ^{12}C-sulfamethazine, if equilibrium is established before extraction starts. The added sulfapyridine in the TLC-procedure only approximates the same recovery as sulfamethazine, due to the slightly different physico-chemical behavior of the molecules in the complex matrix. The recovery for sulfamethazine is 53,6% (n=3) and 33,6% (n=3) for sulfapyridine (both on the 0,10 ppm level).

The TLC-procedure was chosen for the control programme because its results are comparable to the GC-MS method and because it is the most easy to handle.

Table 2 shows the methods for blood analysis and comparable results are seen. Unfortunately, we have not analysed all the blood samples by HPLC at this moment. (The results will however be present on the poster).

Most often the ELISA-test indicates higher sulfamethazine concentration than does the HPLC-method. This is due to the fact that the antibodies in the ELISA-kit react with both sulfamethazine and with the metabolite N^4-acetylsulfamethazine. The HPLC-method is able to distinguish between the two. The difference is also reflected in the standard deviation.

For screening purposes the ELISA-kit is suitable. Actually it provides an extra security to catch all violative samples.

Table 3 shows the blood/muscle ratio for sulfamethazine content. The ratios are calculated from the averages given in table 1 and 2 when ever possible. The results in table 3 validate the 4-factor between blood and muscle concentration as shown in the study of Randecker et al. (1). It is worth noticing that all muscle concentrations are 0,06 ppm or higher. The factor is expected to change when the concentration decreases making it important to maintain a 0,20 ppm limit for sulfamethazine content in blood.

REFERENCES

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Table 1. Results for muscle analysis

Pig number	TLC-method		CG-MS-method (ppm)	Between methods	
	Individual results (ppm)	Average (ppm)		Average (ppm)	SD (ppm)
1	0.79; 0.80	0.80	0.741	0.77	0.04
2	0.10; 0.11	0.11	0.098	0.10	0.009
3	<0.05		0.043		
4	0.08; 0.07	0.08	0.053	0.07	0.019
5	<0.05				
6	0.06; 0.06	0.06	0.015	0.04	0.03
7	<0.05		0.015		
8	<0.05		0.055		
9	0.30; 0.31	0.31	0.304	0.31	0.004
10	0.05;>0.05(too small peak)	0.05	0.060	0.06	0.007
11	0.07; 0.08	0.08	0.044	0.06	0.026
12	0.10: 0.10	0.10	0.065	0.08	0.025
13	0.08; 0.07	0.08	<0.025		
14	0.06; 0.06	0.06	0.056	0.06	0.003
15	0.34; 0.32	0.33	0.253	0.29	0.05

Table 2. Results for blood analysis

Pig number	HPLC method		ELISA-test		Between methods	
	Individual results (ppm)	Average (ppm)	Individual results (ppm)	Average (ppm)	Average (ppm)	SD (ppm)
1	2.05; 2.02	2.04	2.91; 2.93	2.92	2.48	0.6
2	0.30; 0.37	0.34	0.39; 0.50	0.45	0.40	0.08
3	0.16; 0.18	0.17	0.30; 0.24	0.27	0.22	0.07
4	0.17; 0.15	0.16	0.35; 0.31	0.33	0.25	0.1
5	0.11; 0.16	0.14	0.10; 0.12	0.11	0.13	0.02
6			0 0	0**		
7			0.02*; 0.01*	0.02*		
8			0.03*; 0.03	0.03*		
9			1.23; 1.23	1.23		
10			0.19; 0.21	0.20		
11			0.19; 0.21	0.20		
12			0.56; 0.50	0.53		
13			0.39; 0.42	0.41		
14			0.28; 0.23	0.25		
15			1.12; 1.23	1.18		

*

** ekstrapolation
the absorption is equal to or a little higher than adsorption of the sulfamethazine negative standard.

Table 3. The Ratios

Pig number	Muscles (ppm)	Blood (ppm)	Ratio = $\frac{\text{sulfamethazine in blood}}{\text{sulfamethazine in muscles}}$
1.	0.77	2.48	3.2
2.	0.10	0.40	4.0
3.	-	0.22	-
4.	0.07	0.25	3.5
5.	-	0.13	-
6.	0.04	-	-
7.	-	-	-
8.	-	-	-
9.	0.31	1.23	4.0
10.	0.06	0.20	3.3
11.	0.06	0.20	3.3
12.	0.08	0.53	6.6
13.	-	0.41	-
14.	0.06	0.25	4.2
15.	0.29	1.18	4.1