Liquid Chromatographic Analysis of Sulfonamides in Foods

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Key Words

Column liquid chromatography
Sulfonamides in foodstuffs
Honey
Milk
Eggs

Summary

A procedure for the simultaneous determination of several sulfonamides in different foods, such as honey, milk and eggs is proposed. The analysis is carried out using reversed phase liquid chromatography with spectrophotometric detection. Optimization of the mobile phase led to good separation and a short analysis time when an initial isocratic step with a 3 : 97 acetonitrile : water mixture was used for 5 minutes, followed by a linear gradient up to a 40 : 60 mixture over 15 min. The proposed method is suitable for routine quality control analysis to ensure the absence of sulfonamides in foods. Recovery studies yielded good results for all food samples because there were no interferences from the matrices.

Introduction

Sulfonamides are used therapeutically and as animal growth stimulants. Residues are sometimes found in food and, in sufficiently high quantities, are dangerous for human health. Consequently, rapid methods to detect and quantify such residues to evaluate the safety of various kinds of food are required. Most of the reported methods depend on spectrophotometric and chromatographic techniques. Several procedures have been used to determine sulfonamides in milk [1–5], and eggs and animal tissues [6–12]. One of the most frequent diseases in bees is caused by the *Bacillus l*[illegible]*rvae*. This is an extremely contagious disease and can result in the death of the infected bees. Sulfonamides, when administed at controlled concentrations, are harmless to the bees and do not contaminate the honey. However, if very high doses are used, the product can appear in the honey and so it is necessary to check for any possibility of contamination. Several methods for determining sulfonamides in honey have been proposed [13–21].

In this paper, the separation of five sulfonamides which differ in the heterocyclic ring bound to the nitrogen atom and which consequently show different antibacterial activity, is studied. The drugs studied are sulfaguanidine (SG), sulfadiazine (SD), sulfathiazole (ST), sulfapyridine (SP) and sulfamethoxazole (SM). The procedure can be applied to different types of foods such as honey, milk and eggs. Reversed phase liquid chromatography (HPLC) with spectrophotometric detection and a combination of both an isocratic step and a linear gradient is used to obtain optimal separation. A comparison of the proposed method with other existing methods shows advantages as regards simplicity and selectivity. The minimal sample preparation leads to considerably reduced analysis times. Linearity, precision and recovery are also satisfactory.

Experimental

Apparatus

The HPLC system consisted of a Kontron 325 liquid chromatograph operating at room temperature and with a flow-rate of 1 ml/min. A Perkin Elmer LC-85B spectrophotometric detector at a wavelength of 260 nm for all the sulfonamides was used. Aliquots (50 µl) were injected manually using a 7125–075 Rheodyne injector valve. A Hewlett-Packard HP 3396 recording integrator was used to record the chromatograms and integrate the areas under the peaks. The column was of 15 × 0.46 cm i.d. stainless steel packed with 5 µm Spherisorb ODS-2. A Supelco guard column packed with the same stationary phase was also used.

Reagents

Methanol, acetonitrile and ethyl acetate (Romil Chemicals, Loughborough, Leicester, UK) were of liquid chromatographic grade. Doubly distilled water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). The solvents were degassed by purging with helium.

All sulfonamides were obtained from Sigma (St. Louis, MO., USA). Solutions were prepared from the com-

0009-5893/95/04 0382–05 $ 3.00/0

mercial products, without further purification, by dissolving 50 mg in 100 ml of methanol and stored in dark bottles in the cold. Working solutions were obtained by dilution with water immediately before the measurements and kept in the dark.

Calibration Graphs

The separation was carried out with an initial isocratic mobile phase of 3 : 97 acetonitrile-water mixture for 5 min followed by a linear gradient from 3 : 97 to 40 : 60 over 15 min. Finally, the initial conditions were re-established in 1 min and held for 10 min. The flow-rate was 1 ml/min. Other chromatographic conditions were: room temperature; sample-loop, 50 µl; UV wavelength, 260 nm. Under these conditions, linear calibration graphs from 0.05 to 5 µg/ml for all the sulfonamides were obtained. Calibration graphs were prepared by plotting concentration against peak area.

Determination of the Sulfonamides in Foods

Honey. A 1 g honey sample was accurately weighed and dissolved in water in a 10 ml calibrated flask. The solution was homogenized, filtered through a 0.45 µm Millipore filter and injected into the chromatograph. From the peak area value and using the calibration graph, the concentration of the drug was calculated.

Milk and eggs. A sample of 5 ml of milk or 0.4 g of lyophilized egg was treated with 10 ml of trichloroacetic acid solution to give a final concentration of 3 % acid. After homogenization and centrifugation at 5000 RPM for 5 min, the aqueous phase was recovered and the residue re-extracted with 3 % trichloroacetic acid (10 ml). The aqueous phases were combined and diluted to 25 ml with trichloroacetic acid in a calibrated flask. The extract was filtered and injected into the chromatograph.

The incomplete elution of some sample components would contribute significantly to the steady deterioration of the chromatographic column. To prevent this, the column was washed with a 95 : 5 ethyl acetate : acetonitrile mixture at the end of each day.

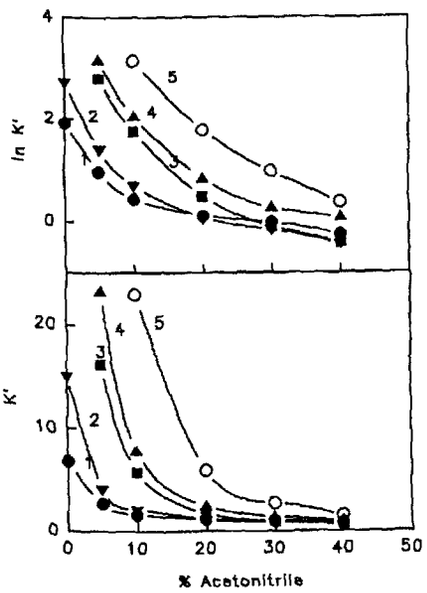

Figure 1

Variation of the retention factors of the sulfonamides with the acetonitrile proportion in the mobile phase. 1, SG; 2, SD; 3, ST; 4, SP; 5, SM.

Results and Discussion

Chromatography of Sulfonamides

The best separation mode was selected by a solubility test. Sulfaguanidine is soluble in water while the other sulfonamides are only slightly soluble in water, insoluble in hexane and freely soluble in methanol. Consequently, reversed phase liquid chromatography was chosen. Several organic modifiers (methanol, acetonitrile, dichloromethane) were tried for mixing with the polar component of the eluent (water). Acetonitrile was chosen since it is a good solvent of the drugs and permitted the column pressure to be kept low. As the proportion of acetonitrile in the mobile phase increased, the retention of the sulfonamides decreased. Figure 1 shows the variation of the retention factors (k) with the different mobile phases. A quadratic relationship between ln k and the fraction of acetonitrile (ϕ) was verified. Both separation parameters, the separation factor (α) and resolution (R_s) increased as the proportion of water increased. Flow rates in the range 0.5–3.0 ml/min were tried and 1 ml/min was selected as a compromise between good separation of the peaks and analysis time.

Proportions of acetonitrile higher than 10 % were required to elute ST, SP and SM adequately. However, these mobile phases led to elution of SG and SD very close to the void volume. Thus, a gradient elution technique was tried to improve the separation. Linear solvent strength programmes were used and the appropriate gradient (initial and final conditions and the transition curve between them) was studied. It was necessary to use a very small percentage of acetonitrile in the initial mobile phase composition, ϕ_0, to avoid elution of SG at the void time. Table I shows some of the gradients tried which gave good separations. The most difficult peaks to separate were those of ST and SP, the difference between the retention times of both compounds being greater when the slope of the gradient decreased. In all cases, blank gradients were performed showing no variations of the baseline. Once several possible gradients had been chosen, chromatographic analysis of different foods was carried out to assay possible interferences from the complex matrix. When a honey sample was injected using one of the selected gradients, numerous peaks appeared at about 4–6 minutes. These peaks, which are characteristic of the honey matrix, interfered with the correct quantifi-

Table I. Effect of the gradient parameters on retention of the sulfonamides.

ϕ_0	ϕ_f	t_s	ϕ' (%/min)	t_R, min SG	SD	ST	SP	SM
0	20	15	1.33	6.39	11.45	13.53	13.99	16.54
0	20	10	2.00	5.87	9.68	11.17	11.54	13.77
0	40	10	4.00	5.49	7.76	8.43	8.73	9.94
2	30	10	2.80	4.84	7.90	8.88	9.24	10.59
5	20	15	1.00	3.97	8.39	11.02	11.59	13.78
5	40	15	2.33	4.02	6.89	8.27	8.69	10.05
5	30	10	2.50	4.02	7.00	8.32	8.63	10.39
5	40	10	3.50	4.34	6.60	7.21	7.46	9.24
5	30	5	5.00	3.35	5.63	6.32	6.62	7.26

Table II. Calibration graphs for the determination of sulfonamides.

Sulfonamide	Slope. 10^5/count. ml/µg	Intercept. 10^5/counts	Correlation coefficient	Detection limit/µg/ml	RSD/%
SG	4.783	0.094	0.9998	0.08	5.6
SD	4.482	- 0.038	0.9999	0.03	3.8
ST	3.588	0.036	0.9998	0.07	4.4
SP	4.339	0.035	0.9998	0.05	3.2
SM	3.832	0.030	0.9999	0.04	5.5

Table III. Slopes of standard additions calibration graphs (countsx10^5).

Sulfonamide/ Sample	Aqueous	Honey	Milk	Egg
SG	4.783	4.624	4.621	4.416
SD	4.482	4.569	4.398	4.383
ST	3.588	3.241	3.459	3.329
SP	4.339	4.186	4.508	4.098
SM	3.832	3.294	3.461	3.225

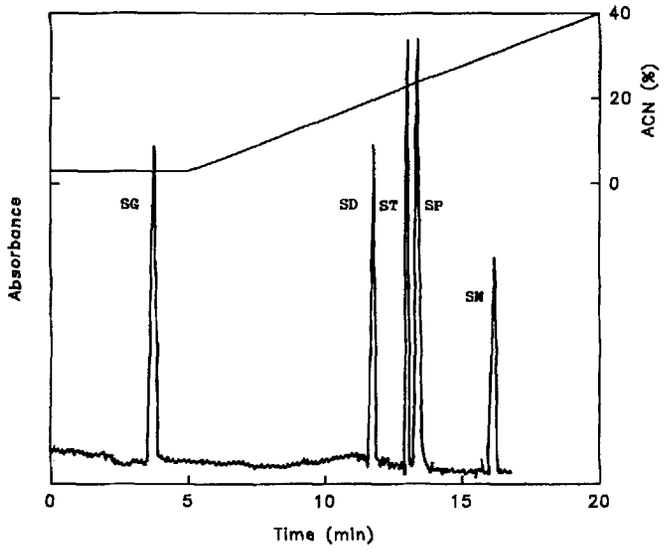

Figure 2

Chromatogram of a mixture containing 0.2 µg/ml of each sulfonamide using gradient elution. Profile of solvent system is shown at the top of the figure. 0.025 AUFS.

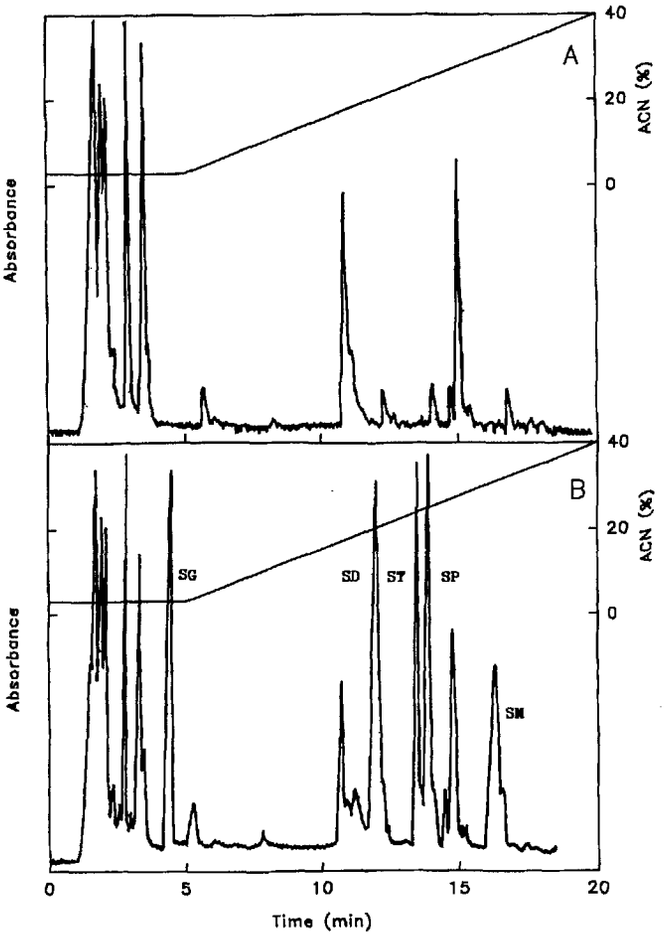

Figure 3

Elution profiles of honey with the optimised gradient elution profile. A, honey; B, honey spiked with 0.2 µg/ml of each sulfonamide. Profile of solvent system is shown at the top of the figure. 0.025 AUFS.

Table IV. Recovery of sulfonamides from food samples.

Food	Sulfonamide	Added, µg/ml	Found, µg/ml	Recovery, %
Honey	SG	2.54	2.37	93.3
		1.01	0.91	91.0
		0.51	0.46	90.2
	SD	2.49	2.49	100.0
		1.00	0.95	95.0
		0.50	0.51	102.0
	ST	2.78	2.55	91.7
		1.11	1.10	99.1
		0.55	0.52	94.5
	SP	2.53	2.53	100.0
		1.01	1.07	105.9
		0.51	0.56	109.8
	SM	2.48	2.24	90.3
		0.99	0.90	90.9
		0.49	0.44	89.8
Milk	SG	2.54	2.52	99.2
		1.01	0.98	97.0
		0.51	0.50	98.0
	SD	2.49	2.49	100.0
		1.00	1.01	101.0
		0.50	0.49	98.0
	ST	2.78	2.69	96.8
		1.11	1.07	96.4
		0.56	0.56	100.0
	SP	2.53	2.35	92.9
		1.01	0.91	90.1
		0.50	0.46	92.0
	SM	2.48	2.36	95.2
		0.99	0.90	90.9
		0.49	0.46	93.9
Egg	SG	2.70	2.55	94.4
		1.35	1.31	97.0
		0.67	0.61	91.0
	SD	2.00	1.99	99.5
		1.00	1.00	100.0
		0.50	0.49	98.0
	ST	2.67	2.57	96.3
		1.34	1.36	101.5
		0.67	0.72	107.4
	SP	2.40	2.26	94.2
		1.20	1.17	97.5
		0.60	0.61	101.6
	SM	2.10	2.08	99.0
		1.05	1.15	109.5
		0.53	0.58	109.4

cation of the sulfonamides and so, a different strategy was tried. The chromatogram was started using an isocratic elution with a mobile phase of 3 : 97 acetonitrile : water for 5 min. In this way, non-retained matrix peaks were eluted followed by the SG peak. Then, the concentration was raised to a 40 : 60 acetonitrile : water mobile phase over 15 min, permitting the elution of SD, ST, SP and SM with the absence of interferent peaks. Figure 2 shows the chromatographic separation of a standard solution of the sulfonamides using the gradient elution system proposed. In this instance, all the sulfonamides considered were well separated in 17 minutes with sharp and symmetrical peaks.

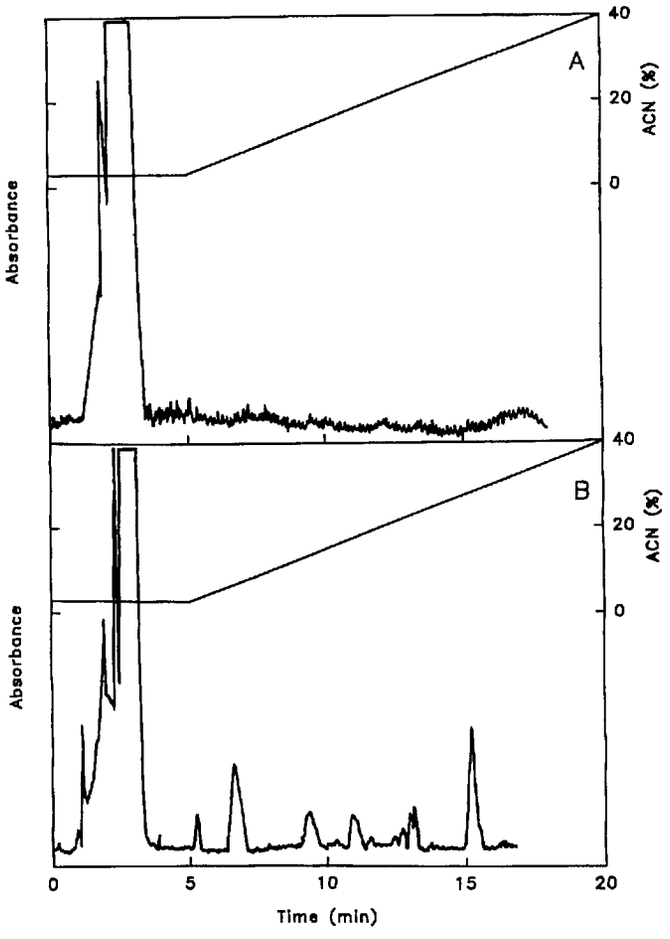

Figure 4
Chromatograms of unspiked milk (A) and egg (B) samples. Profile of solvent system is shown at the top of the figure. 0.025 AUFS.

Calibration, Detection Limits and Repeatability

Calibration graphs were obtained by plotting peak area against concentration and were linear in the range 0.05–5 µg/ml (3–250 ng) for all the sulfonamides. Table II gives the equations for the straight lines obtained and the regression coefficients. The detection limits were calculated on the basis of 3 σ. The precision of the procedure was obtained from the relative standard deviations (RSD) calculated for ten replicate injections of 0.5 µg/ml of each sulfonamide. These values are also given in Table II. Calibration graphs were also obtained in 3 % trichloroacetic acid. Similar results to those achieved in water were obtained for linearity, sensitivity, detection limits and reproducibility.

Recovery Study and Analysis of Commercial Samples

Samples of foods were used to test the method. The absolute recoveries were evaluated by comparing the concentrations found in food samples spiked with known amounts of each analyte to the concentrations found in solution. To investigate the possibility of

interferences caused by the food matrix, the method of standard additions was used. Each graph was constructed from four points and each point represented the mean of three injections. Table III shows that the slopes of the standard additions calibration graphs were similar to those for the aqueous standards, confirming that the food matrix did not introduce interference and that calibration can be carried out with aqueous standards. Food samples were fortified at three concentrations and were extracted as described previously. The absolute peak areas from the extracted samples were compared to standard solutions. The overall mean recoveries which were all greater than 90 %, are shown in Table IV.

The applicability of this proposed method to the measurement of sulfonamides in foods such as honey, milk and eggs, was examined. These products could contain residues of the drugs, making them a potential hazard to public health. The chromatogram obtained from a sample of honey is shown in Figure 3A. Figure 3B presents the chromatogram obtained from a spiked honey fortified with a concentration of 0.2 µg/ml of each sulfonamide considered. The chromatograms showed large peaks near the void volume being well separated from the sulfonamide peaks and other smaller interfering peaks which were not totally resolved from SP and SD. These were equivalent to 0.03 or 0.04 µg/ml of SP or SD, respectively, in honey. The chromatogram also showed a small peak near the retention time of SM; however, peak areas appeared well separated confirming that the assay was sufficiently selective. Figure 4 shows the chromatograms obtained for the analysis of milk and egg samples. Attempts to improve the sensitivity are currently being made. All the food samples analysed were free of sulfonamides above the detection limit.

Conclusion

The proposed HPLC method for the direct determination of sulfonamides in foods provides good separation, reproducibility and recoveries. Sample manipulation is very simple and the complex food matrix does not interfere with the determination. The method may be suitable for application to the quality control of food products.

Acknowledgements

Financial support from Spanish DGICYT (PB93-1138) is gratefully acknowledged.

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Received: Jul 14, 1994
Revised manuscript received: Sep 23, 1994
Accepted: Feb 6, 1995