ELSEVIER

Journal of Chromatography A, 726 (1996) 125–131

JOURNAL OF CHROMATOGRAPHY A

Determination of sulphonamides in foods by liquid chromatography with postcolumn fluorescence derivatization[1]

P. Viñas, C. López Erroz, N. Campillo, M. Hernández-Córdoba*

Department of Analytical Chemistry, Faculty of Chemistry, University of Murcia, E-30071 Murcia, Spain

First received 17 May 1995; revised manuscript received 13 September 1995; accepted 21 September 1995

Abstract

A postcolumn fluorimetric derivatization procedure for the detection and simultaneous determination of several sulphonamides using liquid chromatography was developed. The derivatization method involves the reaction of the sulpha drugs with *o*-phthaldialdehyde and β-mercaptoethanol. The chromatographic elution programme consisted of an initial isocratic step with acetonitrile–water (3:97) for 5 min followed by a linear gradient up to a 40:60 mixture over 15 min. The proposed method was applied to routine quality control analysis to ensure the absence of sulphonamides in foods, resulting in an overall mean recovery of 95% ($n = 45$) at fortification levels of 0.04, 0.1 and 0.5 μg/ml.

Keywords: Food analysis; Derivatization, LC; Sulphonamides

1. Introduction

Sulphonamide residues are frequently found in foods at very low concentrations and liquid chromatographic techniques (HPLC) have been widely used for their determination [1]. Recently, we proposed a new method for determining these drugs using HPLC and spectrophotometric detection [2]. However, the detection limits were not very low and it was thought that one way to improve sensitivity would be to use derivatization reactions. The use of on-line techniques to introduce the derivatization reagents in combination with HPLC improves both selectivity and reproducibility by performing the reaction steps automatically [3]. Several papers concerning spectrophotometric [4–6] or fluorimetric [7–9] derivatization have been published. For the fluorescent detection of sulphamethoxazole, the reagent *o*-phthaldialdehyde (OPA) has been used in combination with β-mercaptoethanol (ME) using flow injection analysis [10]. However, to our knowledge, this derivatization reaction has never been applied to the chromatographic separation of sulphonamides.

In this paper, the separation of the sulphonamides sulphanilamide (S), sulphaguanidine (SG), sulphadiazine (SD), sulphapyridine (SP) and sulphamethoxazole (SM) using reversed-phase liquid chromatography is proposed. Post-column fluorescence derivatization using the re-

[1] Paper presented at the *XXIVth Annual Meeting of the Spanish Chromatography Group. 7.as Jornadas de Análisis Instrumental, April 3–6, 1995, Madrid, Spain.*

* Corresponding author.

0021-9673/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(95)01096-3

agents OPA and ME makes it possible to lower the detection limits of these compounds. The procedure was applied to different types of foods such as trout tissue, egg and milk. Minimal sample preparation leads to considerably reduced analysis times, while the linearity, precision and recovery remain satisfactory.

2. Experimental

2.1. Apparatus

The HPLC system consisted of a Model 325 liquid chromatograph (Kontron, Zürich, Switzerland) operating at room temperature and with a flow-rate of 0.5 ml/min and a Kontron SFM 25 fluorescence detector set at excitation and emission wavelengths of 302 and 412 nm, respectively. Aliquots (50 μl) were injected manually using a Model 7125-075 injection valve (Rheodyne, Berkeley, CA, USA). A PC integration pack (Kontron) was used to record the chromatograms and integrate the areas under the peaks. The analytical column was 15×0.46 cm I.D. stainless steel packed with Spherisorb ODS-2 with a particle size of 5 μm (Teknokroma, Barcelona, Spain). A Supelco guard column packed with the same stationary phase was also used. Homogenization of the samples was achieved by using a T25 Ultra-Turrax homogenizer (IKA, Staufen, Germany), except when milk was analysed, for which manual homogenization tubes of 10 ml equipped with PTFE plungers proved adequate.

The postcolumn reaction equipment consisted of a Minipuls HP4 peristaltic pump (Gilson, Villiers-le-Bel, France), a Hellma 176.052-QS fluorimetric flow cell (Teknokroma, Barcelona, Spain), 0.8 mm I.D. PTFE tubing and various end fittings and connectors (Omnifit, Cambridge, UK). Thermostating of the reactor coil was carried out using a laboratory-made electronic device.

2.2. Reagents

Methanol, acetonitrile and ethyl acetate (Romil Chemicals, Loughborough, 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.

Sulphanilamide, sulphaguanidine, sulphadiazine, sulphapyridine and sulphamethoxazole were obtained from Sigma (St. Louis, MO, USA). Solutions were prepared from the commercial products, without further purification, by dissolving 20 mg in 100 ml of ethanol and stored in dark bottles at 4°C. Working solutions were obtained by dilution with 3% trichloroacetic acid (Sigma) immediately before the measurements and kept in the dark. A 0.01 *M* solution of *o*-phthaldialdehyde (OPA) (Fluka, Buchs, Switzerland) and a 0.02 *M* solution of β-mercaptoethanol (ME) (Sigma) were prepared by dissolving the compounds in 2% ethanol and 0.7 *M* phosphoric acid and kept in dark bottles at 4°C.

2.3. Calibration graphs

Separation was carried out with an initial isocratic mobile phase of acetonitrile–water (3:97) 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 0.5 ml/min. The postcolumn flow manifold is shown in Fig. 1. Derivatization reagents (0.01 *M* OPA and 0.02 *M* ME, both dissolved in 2% ethanol and 0.7 *M* phosphoric acid) were separately added by means of a peristaltic pump at 0.25 ml/min. They were mixed with the column eluent in a four-way connector and reacted in a PTFE coil (2.5×0.8 mm I.D.) thermostated at 40°C using an electronic device. Derivatization reagents were continuously purged with the gas to prevent bubbles appearing during the chromatography. Under these conditions, linear calibration graphs of concentration vs. peak area from 0.01 to 2 μg/ml for sulphamethoxazole and from 0.02 to 2 μg/ml for the other sulphonamides were obtained.

2.4. Sample preparation

2.4.1. Milk

A sample of 3 g of milk was accurately

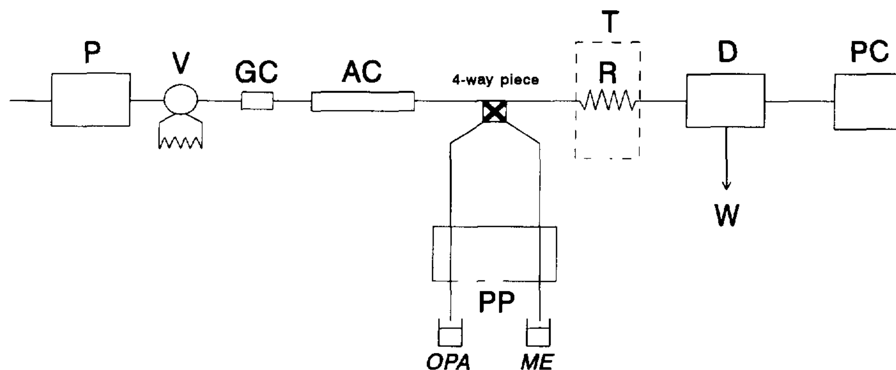

Fig. 1. Manifold for the liquid chromatography of sulphonamides using postcolumn derivatization. P = HPLC pump, flow-rate 0.5 ml/min; V = injection valve; GC = guard column; AC = analytical column; PP = peristaltic pump, total flow-rate 0.5 ml/min; R = reaction coil, 2.5 m × 0.5 mm I.D.; T = electronic thermostat at 40°C; D = fluorimeter (excitation at 302 nm, emission at 412 nm); PC = personal computer; W = waste.

weighed and homogenized in a 10-ml volumetric tube with 0.5 ml of 30% trichloroacetic acid solution. After centrifugation at 5000 rpm for 5 min, the aqueous phase was recovered and the residue re-extracted with 3% trichloroacetic acid (4 ml). The aqueous phases were combined and diluted to 10 ml with trichloroacetic acid in a calibrated flask. The extract was filtered through a 0.45-μm Millipore filter and injected into the chromatograph. From the peak area and using the calibration graph, the concentration of the drug was calculated.

2.4.2. Trout tissue and egg

A sample of 3 g of trout or 4 g of egg was treated with 4 ml of 3% trichloroacetic acid solution. After homogenization with the Ultra-Turrax homogenizer and centrifugation at 5000 rpm for 5 min, the aqueous phase was recovered and the residue re-extracted with 4 ml of 3% trichloroacetic acid. The aqueous phases were combined and diluted to 10 ml with trichloroacetic acid in a calibrated flask. The extract was filtered and injected into the chromatograph.

Incomplete elution of the sample components contributes to the steady deterioration of the chromatographic column, and, to prevent this, the column was washed with ethyl acetate–acetonitrile (95:5) at the end of each day.

3. Results and discussion

3.1. Selection of the mobile phase

As has been previously reported [2], an acetonitrile–water mobile phase is suitable for separating sulphonamides. Preliminary experiments were therefore devoted to checking the chromatographic conditions for using the post-column derivatization procedure. It was found that proportions of acetonitrile higher than 10% were required for a suitable elution of SP and SM, with S, SG and SD being eluted very close to the void volume. This made it necessary to use gradient elution techniques to improve the separation and, since no simple gradient could resolve all sulphonamides, an isocratic elution step coupled with gradient elution had to be used. As recommended elsewhere [2], the chromatogram started with a mobile phase of acetonitrile–water (3:97) for 5 min, in which case S and SG were eluted. The proportions were then changed to acetonitrile–water (40:60) over 15 min, permitting the elution of SD, SP and SM with the absence of interfering peaks. Sulphathiazole gave no fluorescence and, consequently, was not determined.

The mobile phase flow-rate considerably affected the time needed to provide a good analytical signal of the postcolumn reaction. A low

flow-rate increased the fluorescence but resulted in broader peaks, whereas at high flow-rates, the reaction time was too short and severely reduced the detector response. Consequently, an optimum final flow-rate of 1 ml/min in the detector was selected since this clearly separated the chromatographic peaks in an acceptable analysis time. The flow-rate in the HPLC channel was 0.5 ml/min and in the derivatization channels 0.25 ml/min for each reagent.

3.2. Optimization of the postcolumn derivatization

The experimental parameters were optimized to obtain maximum fluorescence signals and Fig. 2 shows the results obtained. As the ME concentration (Fig. 2A) was increased above 0.001 *M*, the peak area increased until a plateau was reached near 0.02 *M* for all the analytes, and this concentration was used in all further work. Variation of the OPA concentration in the 0.001–0.04 *M* range showed maximum fluorescence at a concentration of 0.01 *M* (Fig. 2B). Optimum acidity was selected by adding different amounts of phosphoric acid to both OPA and ME derivatization reagents. Fig. 2C illustrates that the fluorescence reached a maximum value at 0.5–0.7 *M* and then decreased for S, SD and SP, remaining constant for SG and SM. For optimum sensitivity, 0.7 *M* phosphoric acid was added to the reagents. Changes in the reaction coil length between 1 and 6 m also led to a variation of the fluorescence (Fig. 2D). A length of 2.5 m was selected. As indicated above, the flow-rate delivered by both the OPA and ME channels was maintained at 0.25 ml/min to increase the residence time of the sample plug inside the reaction coil, which increased the analytical signal. Heating the reactor coil between 15 and 40°C resulted in an increase in the fluorescence reaction (Fig. 2E). Higher temperatures led to the appearance of bubbles, which distorted the peaks, so 40°C was selected as the optimum temperature.

Fig. 3 shows the chromatographic profile obtained for the mixture of sulphonamides with the derivatization reaction developed. Each peak corresponds to 0.1 μg/ml of sulphonamide.

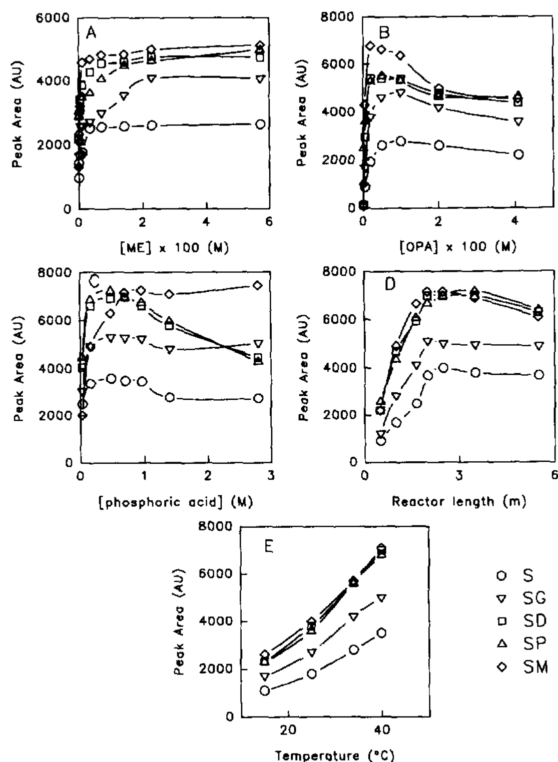

Fig. 2. Influence of the different experimental conditions on fluorescence. (A) β-mercaptoethanol; (B) *o*-phthaldialdehyde; (C) phosphoric acid; (D) reaction coil length; (E) temperature.

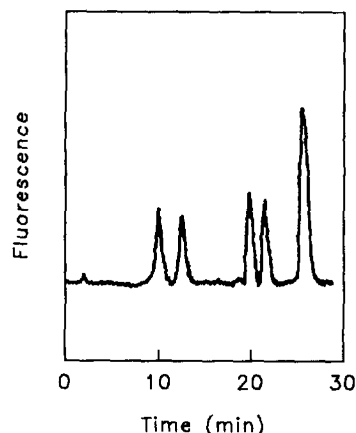

Fig. 3. Elution profile of OPA-derivatized sulphonamides. Each peak represents 0.1 μg/ml. The elution order was S, SG, SD, SP and SM.

Table 1
Calibration graphs for the sulphonamides

Sulphonamide	Slope	Intercept	Correlation coefficient	Detection limit (μg/ml)	R.S.D. (%)
S	66.43	−0.189	0.9998	0.018	6.4
SG	82.88	−0.450	0.9997	0.019	7.9
SD	101.46	−0.189	0.9998	0.016	4.0
SP	103.49	0.120	0.9998	0.013	4.1
SM	253.67	1.340	0.9999	0.011	5.5

3.3. Calibration, detection limits and repeatability

Table 1 shows the equations obtained for the calibration graphs of the sulphonamides and the regression coefficients. The calibration graphs were obtained by plotting peak area against concentration and were linear over the range 0.01–2 μg/ml (0.5–100 ng) for sulphamethoxazole and 0.02–2 μg/ml (1–100 ng) for the other sulphonamides. The detection limits were calculated on the basis of 3σ. The precision of the procedure was obtained from the relative standard deviation (R.S.D.) calculated for ten replicate injections of 0.04 μg/ml of each sulphonamide. The results are given in Table 1.

3.4. Recovery study and analysis of food samples

An analysis of typical food samples in which sulphonamides might occur as residues was carried out. Owing to the high sensitivity of the method, drugs could be detected in the food extracts without the use of any preceding purification step. Fig. 4 shows examples of typical

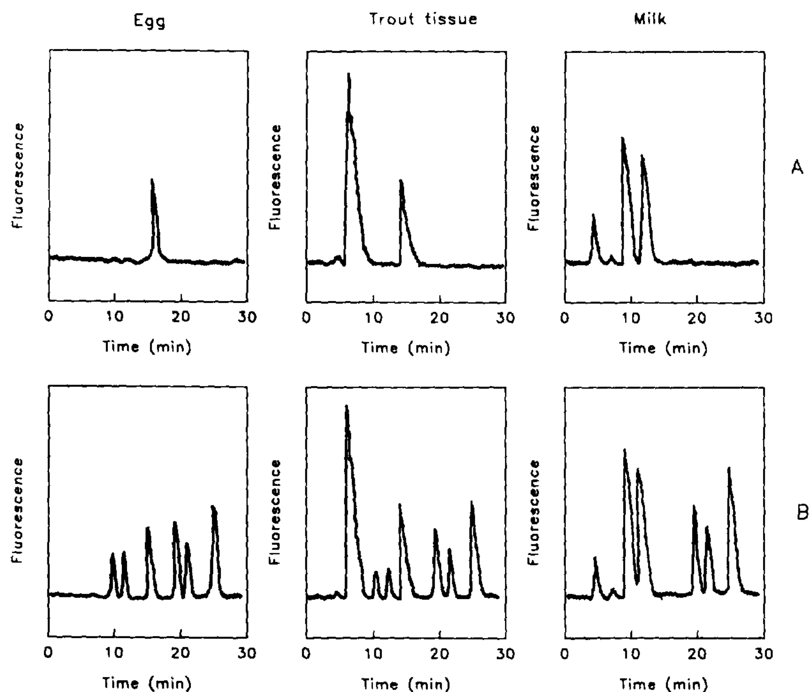

Fig. 4. (A) Typical blank and (B) fortified at 0.1 μg/ml sulphonamide chromatograms for egg, trout tissue and milk samples.

blank (A) and spiked (B) samples. Profiles corresponding to egg and trout tissue showed that sulpha drug peaks are completely separated from each other and from other matrix compounds, any overlap with interfering peaks caused by other compounds being negligible. In the chromatograms obtained for milk samples, two small interfering matrix peaks were observed at about 9.7 and 12.0 min, neither S nor SG being completely resolved. For this reason, the detection limits of S and SG in milk were higher than those found for other samples. All the food samples analysed were free from sulphonamides above the detection limit.

The standard additions method was used to investigate the possibility of interference by the matrix food. Each graph was constructed from four points and each point represented the mean of three injections. Slopes of the standard additions calibration graphs were similar to those of aqueous standards, confirming that the matrix food did not interfere and that calibration can be carried out with the simplest method using aqueous standards. A summary of the slopes and detection limits calculated from each food matrix is given in Table 2. The absolute recoveries were evaluated by comparing the concentrations found in food samples spiked with known amounts of each analyte and the concentrations found in solution. Food samples were fortified at three concentrations (0.04, 0.1 and 0.5 μg/ml) and were extracted as described under Experimental. The mean overall recoveries are shown in Fig. 5. For milk recovery studies, S and SG were only added at the 0.5 μg/ml level. When all spike and recovery data were combined, an average recovery of 95% ($n = 45$) was obtained. This average value indicates that recovery is essentially quantitative.

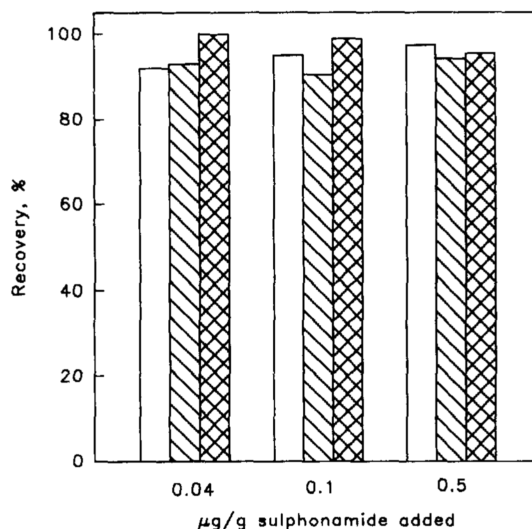

Fig. 5. Recovery of sulphonamides from trout (blank), egg (lines) and milk (crossed lines) samples. Values are the mean recoveries ($n = 5$) of all sulpha drugs in each matrix.

4. Conclusion

The derivatization reaction proposed considerably lowers the detection limits of sulphonamides in complex food matrices and is suitable for joint use with HPLC separation of

Table 2
Slopes and detection limits of sulphonamides in food samples

Sulphonamide	Milk		Egg		Trout	
	Slope	Detection limit (μg/g)	Slope	Detection limit (μg/g)	Slope	Detection limit (μg/g)
S	64.5	0.330[a]	63.2	0.040	61.4	0.060
SG	76.9	0.340[a]	74.3	0.047	83.0	0.063
SD	100.2	0.053	101.4	0.040	91.9	0.053
SP	105.8	0.043	95.8	0.032	96.8	0.043
SM	243.2	0.036	251.4	0.027	248.9	0.037

[a] Detection limits of S and SG were higher owing to the interfering peaks mentioned in the text.

these drugs. The method is sensitive enough to be applied to biological samples. Simple sample preparation and automatic derivatization considerably decrease the total analysis time needed.

Acknowledgements

Financial support from the Spanish DGICYT (Project PB93-1138) and from the Comunidad Autónoma de la Región de Murcia (CARM, PIB94/09) is gratefully acknowledged. N. Campillo acknowledges a fellowship from the Consejería de Cultura, CARM.

References

[1] E. Heftmann, Chromatography, Fundamentals and Applications of Chromatography and Related Differential Migration Methods, Elsevier, Amsterdam, 1992.
[2] P. Viñas, C. López Erroz, A. Hernández Canals and M. Hernández Córdoba, Chromatographia, 40 (1995) 382.
[3] R.P.W. Scott, Liquid Chromatography for the Analyst, Marcel Dekker, New York, 1994.
[4] R.W. Stringham, E.C. Mundell and R.L. Smallidge, J. Assoc. Off. Anal. Chem., 65 (1982) 823.
[5] R.L. Smallidge, E.J. Kentzer, K.R. Stringham, E.H. Kim, C. Lehe, R.W. Stringham and E.C. Mundell, J. Assoc. Off. Anal. Chem., 71 (1988) 710.
[6] M.M.L. Aerts, W.M.J. Beek and U.A.Th. Brinkman, J. Chromatogr., 435 (1988) 97.
[7] H.S. Sista, D.M. Dye and J. Leonard, J. Chromatogr., 273 (1983) 464.
[8] N. Takeda and Y. Akiyama, J. Chromatogr., 558 (1991) 175.
[9] N. Takeda and Y. Akiyama, J. Chromatogr., 607 (1992) 31.
[10] C. López Erroz, P. Viñas and M. Hernández Córdoba, Talanta, 41 (1994) 2159.