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High-performance liquid chromatography of sulphonamides extracted from bovine and porcine muscle by solid-phase dispersion

L. V. Walker*, J. R. Walsh and J. J. Webber

Regional Veterinary Laboratory, Department of Agriculture, P.O. Box 406, Hamilton, Victoria 3300 (Australia)

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ABSTRACT

A method has been developed for the analysis of sulphonamides in bovine and porcine muscle, based on solid-phase dispersion. Muscle tissue was blended with pre-washed C_{18} coated silica (55–105 μm), and the resulting homogeneous solid packed into a polypropylene syringe barrel. Fatty material was washed from the sample using hexane, and the sulphonamide analytes eluted with dichloromethane. The collected fraction was dried under nitrogen and reconstituted in 20% methanol in 0.01 *M* sodium acetate/acetic acid (pH 5) buffer. After sonication and filtration, the sample was analysed by high-performance liquid chromatography on a C_{18} column using UV diode array detection. Individual sulphonamides could be detected down to 0.01 ppm, whilst analyte identity could be confirmed by diode array spectrum down to 0.02 ppm.

INTRODUCTION

The maximum residue limit (MRL) in Australia for sulphamethazine and sulphadiazine in bovine and porcine muscle tissue is 0.1 ppm. A method for sulphonamide extraction from muscle was therefore required which would allow assays in the 0.02–0.05 ppm range, with very low signal-to-noise ratios at the MRL.

Extraction of sulphonamides from tissues traditionally has been performed by any of a large number of variations on liquid–liquid partitioning [1–5], or more recently by liquid extraction followed by solid-phase extraction [6,7]. A new extractive technique has been described, solid-phase dispersion (SPD) [8], and applied to the extraction of sulphonamides from milk [9], pig muscle [10], fish [11] and infant formula [12]. In this method, the sample was dispersed over a large area of C_{18} derivatised silica —typically 0.5 g of tissue over 1000 m^2 of surface area, allowing practically total exposure of the sample to an extracting solvent. The dispersed material is packed into a column (a syringe barrel) and eluted according to a normal-phase chromatographic sequence with a relatively low volume (< 10 ml) of dichloromethane, from which a sample is easily prepared for chromatography.

In the method presented below, the SPD technique has been modified to allow greater sensitivity and higher sample throughput than the original published method [8], applied to the analysis of spiked and incurred sulphonamide residues in bovine and porcine muscle, and then applied in surveillance and monitoring testing programs in Australia.

MATERIALS AND METHODS

Chemicals

Hexane, ethyl acetate (Nanograde; Mallinckrodt, Paris, KY, USA), acetonitrile, methanol, dichloromethane (HPLC grade; Waters, Milford, MA, USA), sulphisomidine, sulphadiazine, sulphathiazole, sulphamerazine, sulphamethazine (Sigma, St.

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Louis, MO, USA), anhydrous sodium acetate (AnalaR grade; BDH, Kilsyth, Australia), acetic acid (analytical-reagent grade; Mallinckrodt).

Disposables

Plastic weighing trays (80 mm × 80 mm × 20 mm); No. 22 scalpel blades (Swann-Morton, Sheffield, UK); C_{18} silica (Waters Preparative C_{18}; 55–105 μm, 125 Å pore size, end-capped, 12% carbon load); 10-ml and 1- or 2-ml polypropylene disposable syringes (Terumo, Melbourne, Australia); filter paper Whatman 541 (Whatman, Maidstone, UK); silanised glass wool; 0.45-μm disposable disc filters with slip Luer fitting (FlowPore D26; Flow Laboratories Australasia, North Ryde, Australia); Luer-Lock needles; 1.25 × 38 mm (Becton Dickinson, Singapore); autosampler vials.

Equipment

Sample preparation. 20- or 50-ml glass syringe barrel, for washing C_{18} solid phase; Sample evaporator (Turbovap; Zymark, Hopkinton, MA, USA); ultrasonic bath (FX-8; Unisonics, Sydney, Australia).

Liquid chromatograph. Varian Star System (Varian, Walnut Creek, CA, USA), comprising a 9010 ternary pump, 9065 diode array UV spectrophotometer (190–367 nm range), 9095 autosampler (6-port Valco, 1-ml loop), data handling and system control PC (Varian Star software).

LC columns. C_{18} Novapak 100 mm × 8 mm Radial-Pak cartridge (end-capped, 4-μm particle, 60 Å pore, 7% carbon load) and μBondapak C_{18} guard column (end-capped, 6–12 μm, 125 Å, 11% carbon load) (Waters).

Chromatographic conditions

LC solvents. (A) 0.01 *M* sodium acetate, buffered to pH 5 with acetic acid; (B) 100% acetonitrile.

LC conditions. Flow-rate, 2.4 ml/min. Start, 100% A; 8 min, A–B (60:40); 12 min, A–B (60:40); 15 min, 100% A; 20 min, 100% A.

Preparation of solid phase

Bulk C_{18}-coated silica (C_{18}) was washed in twice its own volume of hexane, dichloromethane, ethyl acetate and methanol (in succession) by drawing the solvent through a glass syringe packed with C_{18}. The C_{18} was air-dried after the methanol wash, and has an indefinite shelf life once sealed away.

Sample preparation

For successful assay of a muscle sample for sulphonamides, the tissue must be of high quality. The assay required meat trimmed free of fat and connective tissue.

Slivers of trimmed frozen muscle were then shaved off using a scalpel, and transferred directly to a plastic weighing tray for a final sample mass of 1 g. At this stage, the sample was either spiked with sulphonamides for a recovery assay, or spiked with 100 μl of 1 μg/ml sulphisomidine in methanol as an internal standard. To this was added 2.5 g of the washed and dried C_{18}. Each sample was then ground into a homogeneous solid, using a test tube as the pestle. The blending was performed using a pressing and light grinding action, rather than an outright grinding action, to minimise crushing of the silica. The blended material was shredded using the pestle and a spatula and then packed into 10-ml polypropylene syringe barrels which had the outlets plugged with silanised glass wool. The sample was topped off with a disc of filter paper and then compressed down to approximately 4 ml, using the syringe plunger from which the rubber end and plastic support had been cut off to give a flat-ended plunger.

The column was then filled to the top with hexane, which was either allowed to drain through, or aspirated through under a mild vacuum. Air was then blown through or a mild vacuum applied to the column to remove excess hexane and give a dry-appearing sample. The column was then filled to the top with dichloromethane, and again either aspirated or allowed to drain directly into a 50 ml Turbovap tube, until the column appeared dry. The collected fraction was reduced to dryness in the Turbovap under dry nitrogen. The dried extract was then reconstituted in 200 μl of methanol, vortex mixed, and a further 800 μl of the first chromatographic mobile phase added. The sample was again vortex mixed, and then placed in an icewater-filled ultrasonic bath for 15–20 min. The resulting opaque suspension was drawn up in a 1- or 2-ml disposable syringe, and filtered, through a 25-mm diameter 0.45-μm filter, into HPLC autosampler vials.

Sample analysis

A 300-μl volume sample was injected for each run, although up to 1 ml could be used if desired.

UV absorbance was monitored at 263 nm, with a usable diode array range of 220–367 nm available for confirmation of compounds by their spectral characteristics.

RESULTS AND DISCUSSION

The determination of sulphonamide residues in muscle tissue on a regular basis requires an assay that is fast and relatively cheap and has a high degree of repeatability. To allow other laboratories to adopt the method without great capital outlay, the equipment required for the assay must also be readily available and reuseable, or if not, of low enough cost to be disposable.

To this end the solid-phase dispersion method was examined for use in this assay. Initial work was performed at the 1 ppm level using readily available Waters C_{18} (in the ratios of the initial work [8]). This indicated far higher recoveries than had been found with the more traditional wet extractive methods of analysis: around 90–100% recovery for sulphadiazine, sulphamerazine and sulphamethazine. Further examination revealed only slightly lower recoveries at 0.1 ppm. The process was re-examined in an effort to increase the sample size to 1 g or more, in a final volume of 1 ml. It was found that a sample of 1 g of tissue to 2.5–3 g of C_{18} was readily prepared without loss of recovery. The method as developed allowed the detection and quantitation to 0.01 ppm and diode array confirmation down to 0.02 ppm for sulphadiazine, sulphathiazole, sulphamerazine and sulphamethazine.

Chromatograms of a 0.1-ppm standard, an unspiked muscle and a muscle sample spiked at 0.1 ppm are given in Fig. 1A–C. The spiked muscle gave recoveries (intra-assay) of 85% for sulphadiazine, 37% for sulphathiazole, 80% for sulphamerazine and 76% for sulphamethazine. Results for these muscle samples spiked at 0.1 ppm are given in Table I, along with inter-assay data taken from 12 successive spikes associated with actual assays made with this method. Muscle samples spiked at 0.05 ppm gave recoveries equivalent to those obtained at 0.1 ppm, although the relative standard deviation (R.S.D.) of sulphadiazine and sulphathiazole increased markedly at the lower level. Results for these muscle samples spiked at 0.05 ppm are also presented in Table I. Four replicates are used at

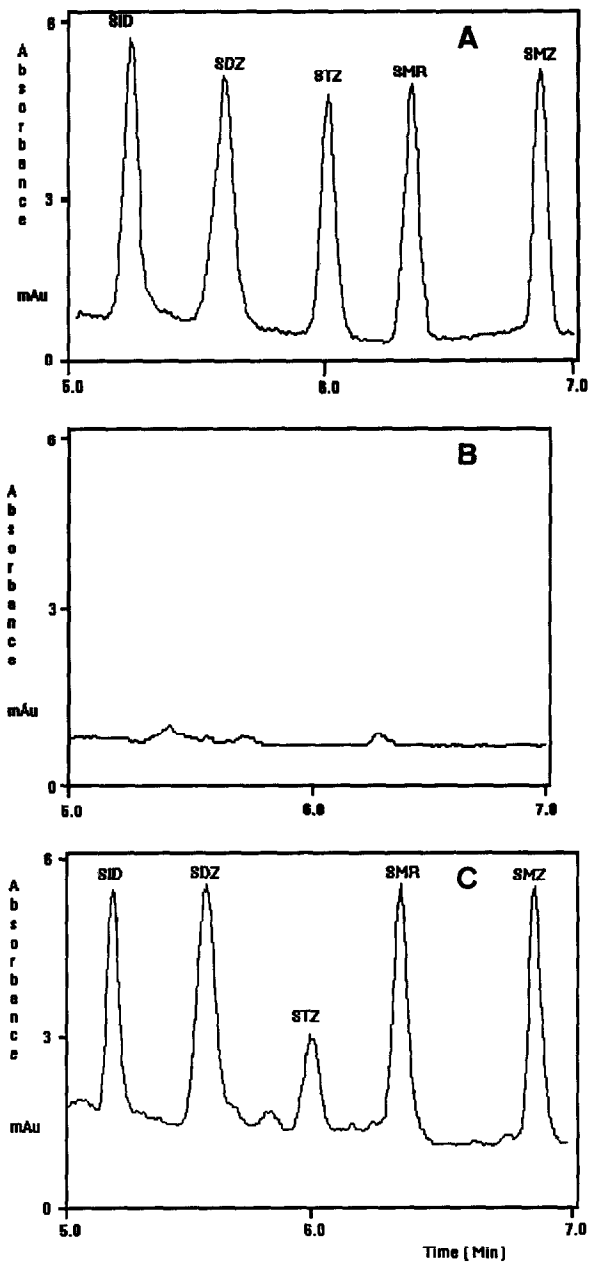

Fig. 1. (A) Chromatogram of 0.1 ppm standard; (B) chromatogram of unspiked bovine muscle; (C) chromatogram of 0.1 ppm spiked bovine muscle. SID = sulphisomidine; SDZ = sulphadiazine; STZ = sulphathiazole; SMR = sulphamerazine; SMZ = sulphamethazine. Chromatographic conditions: Absorbance monitored at 263 nm; gradient 0–40% acetonitrile over 8 min, hold 4 min, 40–0% acetonitrile over 3 min, hold at 100% acetate buffer for 5 min; flow-rate 2.4 ml/min.

TABLE I

INTRA- AND INTER-ASSAY RECOVERY OF SULPHONAMIDE ANALYTES FROM BLANK CALF MUSCLE SPIKED AT 0.1 PPM AND 0.05 PPM

Chromatographic conditions as in Fig. 1.

Analyte	Spike value (ng/g)	Intra-assay (n = 4)			Inter-assay (n = 12)	
		Mean (ng/g)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Spiked at 0.1 ppm						
Sulphadiazine	130	110	85	5	71	10
Sulphathiazole	103	38	37	8	38	31
Sulphamerazine	99	80	80	4	82	16
Sulphamethazine	104	79	76	6	80	10
Spiked at 0.05 ppm						
Sulphadiazine	65	50	77	22		
Sulphathiazole	52	22	42	27		
Sulphamerazine	50	41	82	2		
Sulphamethazine	52	38	73	8		

each spike level, to allow calculation of standard deviation and recovery percentages.

Fig. 2 shows the resulting chromatogram from an animal which was injected with a therapeutic dose of sulphamethazine approximately one week prior to slaughter. The level of sulphamethazine found was 0.10 ppm (corrected for recovery data of Table I). The diode array spectrum for sulphamethazine in the incurred residue sample *vs.* the sulphamethazine spectrum of the standard is given in Fig. 3.

Fig. 4 shows the spectrum of sulphisomidine added to the incurred residue sample *vs.* the sulphisomidine spectrum from the standard. Sulphisomidine, as an internal standard, may be used for calculation of analyte quantities but is also useful as a reference marker in chromatographic runs. The sulphisomi-

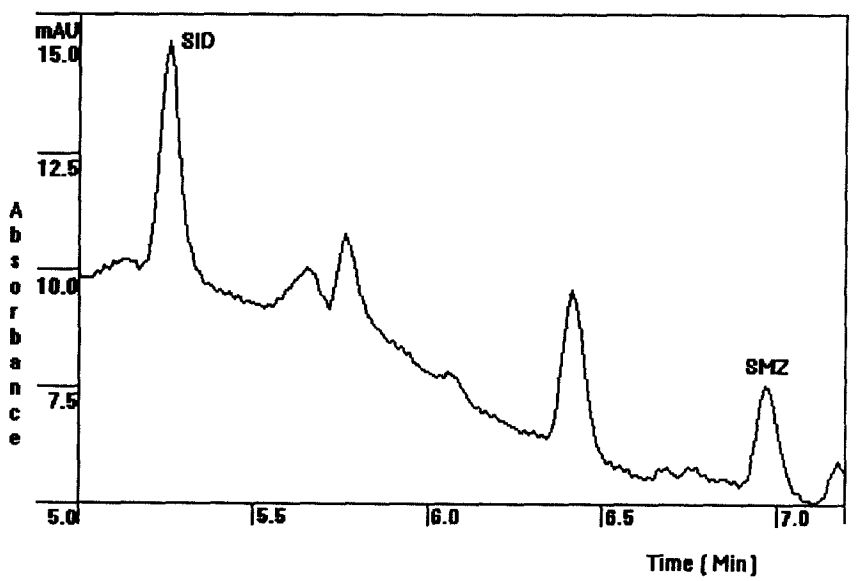

Fig. 2. Chromatogram of incurred sulphamethazine sample. SID = Sulphisomidine; SMZ = sulphamethazine. Chromatographic conditions as in Fig. 1.

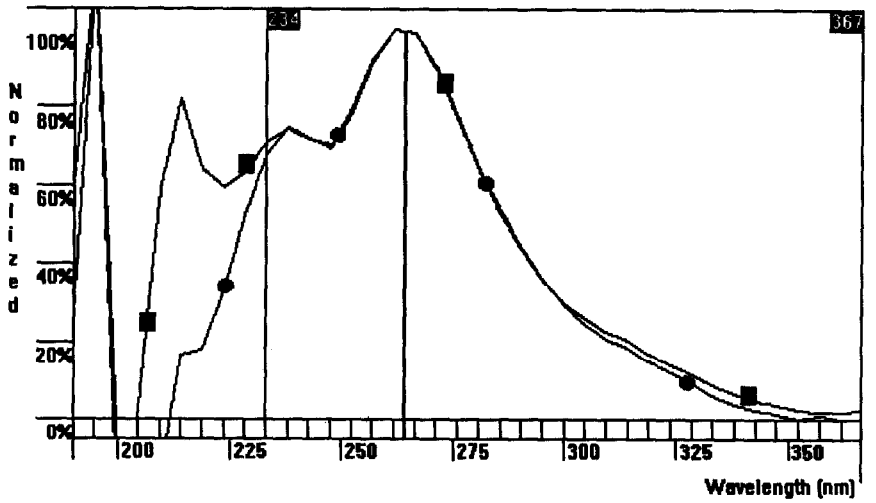

Fig. 3. Diode array spectrum of sulphamethazine from the incurred sample *vs.* sulphamethazine from the standard in Fig. 1A. ■ = Incurred sample; ● = standard.

dine peak may be recognised by its characteristic spectra (see Fig. 4), and used as an indicator for the relative position of the other analyte peaks if the retention times have moved during an extended period of operation (*e.g.*, overnight).

Other C_{18} packings were tried in the course of the method development. Two different 40-μm packings were obtained: Analytichem (originally specified [8]) and Davisil (Alltech, Deerfield, IL, USA). These had the theoretical advantage of larger surface area, giving higher sample loads. In practice it was found that a larger quantity of solid phase was required to form a dry homogenate with the tissue, and the material was generally more difficult to handle and blend than the coarser Waters material. The finer packings tended to "clump" together, and the homogenate was formed when enough C_{18} was added to coat and blend through the tissue matrix. The coarser Waters material tended to cut through and fragment the tissue matrix with significantly

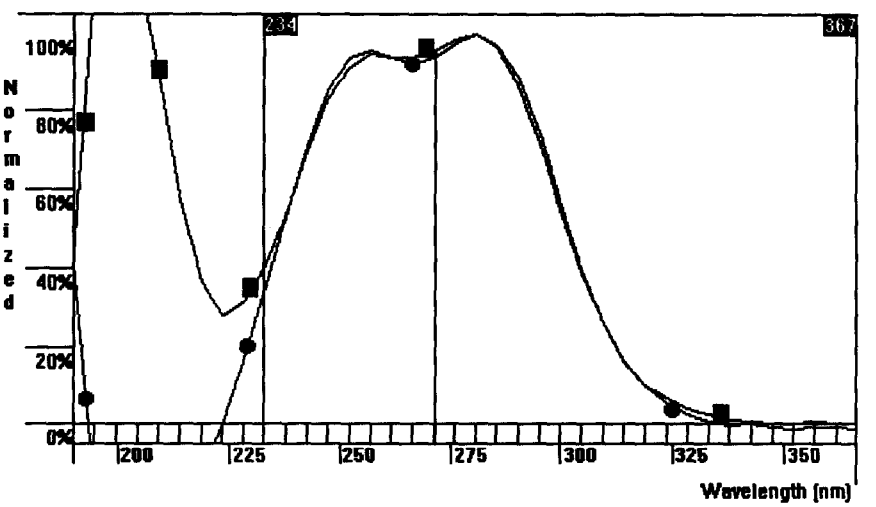

Fig. 4. Diode array spectrum of internal standard sulphisomidine in incurred SMZ sample *vs.* sulphisomidine in the standard of Fig. 1A. ■ = Incurred SMZ sample; ● = standard.

less effort, whilst tending to "clump" less even on very moist tissue. The 40-μm packings proved to be inferior overall, with recoveries well below that obtained with the Waters material. This was found to be caused partly by vacuum cavitation in the packed bed during washing and eluting. Elution under gravity improved the yield, although not to the level obtained with the Waters material. Whilst the SPD method may be used with gravity or vacuum elution, the sample throughput is significantly increased by vacuum elution. The restrictions inherent in using the fine grade C_{18} packings, both from the sample preparation and elution aspects, were felt to be unacceptable.

The SPD method allows for fast sample extraction and clean-up whilst using a minimum of extraction solvent. This technique, unlike traditional solvent extraction procedures, is quite easily expanded to allow a single operator to process multiple samples in one day (up to 20).

A limitation inherent in the method is that of sample size. If a sample much greater than 1 g is used, the sample preparation becomes more time consuming in both blending and eluting. If scaling-up was required, it would actually be easier to prepare the sample in multiple batches of 1 g, and elute all of the columns into the one Turbovap tube for drying. The speed of the method would be seriously jeopardised if more than 5 g of sample were required. Loss of sample homogeneity through directly taking 1 g of a tissue sample has not to date appeared as a problem. The method has performed well in Australian quality assurance programmes, competing successfully against a variety of liquid-extraction and solid-phase extraction methods, all using homogenized muscle samples.

The use of UV diode array detection in the assay allowed for the positive identification of sulphonamide analytes without actually having to re-run the samples in question. The acetate buffer used had significant advantages over similar pH phosphate, citrate or oxalate buffers with respect to the operation and use of a diode array instrument. The detector used had an operational wavelength range from 190 to 367 nm, but absorption from the acetonitrile and the acetate buffer reduced this range to 220–367 nm. The phosphate, citrate and oxalate buffers tended to raise the lower operational wavelength to around 250 nm. As the absorbance maxima for three of the five sulphonamides was 263 nm, spectral information useful or vital for analyte confirmation was lost. Confirmation of an analyte from its 220–367 nm spectrum was readily made against library spectra from samples spiked at 0.1 ppm (such as Fig. 1C). Library referencing could be made either manually (post-run) or as an extension of the automated post-run computing sequence. The library spectra were found to be of more use if taken from chromatograms of spiked samples, as this provided the background which was otherwise missing from pure standards. This also allowed the analyte species to be confirmed down to 0.02 ppm.

The SPD method opens up possible sample sources which would not be considered feasible by the more traditional wet extraction methods. As only 1 g of quality tissue is required, muscle biopsies are possible, and may be of great value in trace-back operations, similar to those followed for organochlorine residues monitoring in Australia. Similarly, the small sample required could allow for random monitoring of carcasses at an abattoir with minimal or no damage to the carcass.

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