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TRACE ANALYSIS OF SULFAQUINOXALINE IN ANIMAL TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Sulfaquinoxaline (in combination with diaveridine as a potentiating agent) was administered orally to broilers, 4—5 weeks old, and sulfonamide residues were determined in muscle and liver at 0, 1, 2, 4, 6, 7, 8, and 10 days post-treatment, using ion-pair extraction followed by high-performance liquid chromatography with UV detection. Improved recoveries (ca. 80%, at the 10 ppb* level) were obtained after liquefaction of the tissues by the addition of 8 *M* urea and sodium hydroxide, prior to ion-pair extraction. A withdrawal period of seven days was found necessary in order to reduce drug residues in muscle and liver to 10 ppb, a level without hazard to humans.

INTRODUCTION

Sulfaquinoxaline and other sulfa drugs are widely used in feeds and premixes to prevent various intestinal disorders of domestic fowl and cattle. As the drugs are stored in tissue and can be ingested by humans, drug manufacturers have since 1973 been required to submit data on tissue residues of sulfonamides following their use in food-producing animals.

In order to quantitate no-effect sulfaquinoxaline (Sq) levels in tissues, methods of marked sensitivity and selectivity are needed. The earlier analytical methods, such as those based on the Bratton-Marshall procedure [1] and subsequent modifications [2—4], failed to meet these requirements due mainly to lack of specificity [5].

Of the methods with improved selectivity, high-performance liquid chromatography (HPLC) proved to be superior to either thin-layer or gas chromatography [6] in terms of simplicity and efficiency. Much work has been carried out on the HPLC separation [7, 8] and quantitative evaluation of various sulfonamides [9—11], but only one paper has been published for the

*Throughout the article the American billion (10^9) is meant.

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determination of Sq in tissues with remarkably low (30 ppb) detection limits [12]. The extraction procedure is, however, rather laborious and running a chromatogram may require over 150 min.

This paper reports the optimization of the extraction procedure of Sq from poultry tissues (muscle and liver), the selection of the appropriate internal standard, and the optimization of HPLC system parameters, with a view to sensitivity. The rate of tissue-residue depletion of Sq is also presented.

EXPERIMENTAL

Materials

Acetone, acetonitrile, *n*-hexane and isoamyl alcohol (each Uvasol), methanol (Selectipur) and ethyl acetate (LiChrosolv) were purchased from Merck (Darmstadt, G.F.R.); chloroform (Chrom AR) was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Ethyl methyl ketone was the product of Reanal (Budapest, Hungary). The sources of the compounds examined were as follows: Sq (Chinoin, Budapest, Hungary); sulfadimethoxine (Sd) (Hoffmann-La Roche, Basel, Switzerland); sulfaguanidine (Sgd), sulfadimidine (Sdd), sulfamethoxidiazine (Smd) (Alkaloida, Tiszavasvári, Hungary); diaveridine (Burroughs Wellcome, Research Triangle Park, NC, U.S.A.). Bis-(2-ethylhexyl)-hydrogen phosphate (DEHP) and trioctylmethylammonium chloride (Adogen 464) were supplied by Serva (Heidelberg, G.F.R.); sodium lauryl sulfate (SLS) was obtained from Schuchardt (München, G.F.R.); tetrabutylammonium hydrogen sulfate (THS) was the product of Sigma (St. Louis, MO, U.S.A.). Other chemicals used were of the highest grade available.

Stock solutions of Sq and Sd were prepared at 0.1 mg/ml in acetone and stored in the refrigerator. For direct calibrations (solutions only, no tissue) varying aliquots of Sq stock solution (10—150 μl) were combined with 100-μl aliquots of Sd stock solution and diluted to 8.0 ml with the eluent. For the residue assay procedure 500 μl and 1000 μl aliquots of Sd stock solution were diluted to 40.0 ml with distilled water containing 1 ml of 25% (w/v) ammonia per 100 ml (solutions A and B, respectively).

Alkaline urea solution (AUS): solutions of 8 *M* urea and 1 *N* NaOH were mixed before use, in a volume ratio of 10:1. Tetrabutylammonium hydrogen sulfate solution (TBAHS): 0.5 g of THS was dissolved in a mixture of 2 ml of isoamyl alcohol and 498 ml of freshly distilled ethyl methyl ketone. The solution was further diluted with 500 ml of freshly distilled ethyl acetate. Trichloroacetic acid solution (TAS): 83 g of trichloroacetic acid were dissolved in 250 ml of distilled water. Borate buffer solution (BBS): 40 g of boric oxide were suspended in 500 ml of distilled water and titrated to pH 11.5 with 10 *N* NaOH. The solution was diluted to 600 ml with distilled water and filtered.

Procedures

Twenty-eight 4—5-week-old broilers were assigned to two groups. Four chickens were fed the standard growing chick mash as a basal control diet (control group). Twenty-four chickens were given the drug as a commercial premix (containing 8 g of Sq and 4 g of diaveridine in 100 kg of premix) for

two weeks before they were given the control diet only (post-treatment). Three chickens from the treatment group were killed on days 0, 1, 2, 4, 6, 7, 8, and 10 post-treatment by decapitation. The control group was sacrificed on the third day of this period. Tissue samples were analyzed without delay.

Residue assay procedure for muscle (procedure A) (see Fig. 1). The muscle was cut into small pieces and 20 g were weighed into the beaker of the homogenizer (MSE, with 100-ml vortex beakers and stainless-steel blades; Measuring and Scientific Equipment Ltd., London, Great Britain). Following the addition of 2.0 ml of solution A, the sample was thoroughly mixed with a glass stirring rod and allowed to stand for 5—10 min. After the addition of 45 ml of AUS, the mixture was homogenized for 5 min, then transferred to a centrifuge bottle and centrifuged at 6000 *g* (MSE, Mistral 4L) for 15 min. Next, the clear liquid was decanted into a 500 ml separating funnel, while the residue in the centrifuge bottle was replaced into the homogenizer, homogenized with another 45-ml portion of AUS and centrifuged as before. The clear liquid was decanted into the 500 ml separating funnel.

The extraction procedure was as follows. The viscous liquid in the separating funnel was mixed with 120 ml of TBAHS and, with frequent shaking, 12 ml of TAS were added in small portions. The resulting suspension was thoroughly shaken for 3—5 min, allowed to stand for 10—15 min and shaken again. The precipitate was removed by filtering the suspension through a suction filter. The filtrate was placed into a 350 ml separating funnel and, after separation, the lower phase was collected in a beaker. The precipitate in the filter was resuspended in the lower phase, the suspension was replaced into the 500-ml separating funnel and, with another 120-ml portion of TBAHS but without additional TAS, the extraction and the filtering processes were repeated.

The cake in the filter was washed with 15—20 ml of TBAHS and the whole filtrate was combined with the upper phase in the 350-ml separating funnel. After separation, the aqueous lower phase was discarded. The organic upper phase was washed by adding 35—40 ml of distilled water, shaking for 3 min and discarding the lower phase. The organic phase was placed in a 250-ml round-bottomed flask and about nine-tenths of the organic solvent was evaporated on a rotating evaporator at a temperature of about 90°C. Using some ethyl acetate, the residue in the flask was quantitatively transferred to a small separating funnel and washed again by the addition of 10—12 ml of distilled water, as described above. The organic phase was placed in a 50-ml round-bottomed flask and evaporated to dryness.

Residue assay procedure for liver (procedure B). The liver was pulped with a knife and 20 g were weighed into the beaker of the homogenizer. Following the addition of 2.0 ml of solution B, the sample was thoroughly mixed with a glass stirring rod and allowed to stand for 5—10 min. After the addition of 70 ml of AUS the mixture was vortexed for 5 min, then transferred to a centrifuge bottle and centrifuged at 6000 *g* for 15 min. The clear liquid was decanted into a 500-ml separating funnel.

The extraction procedure was the same as in procedure A, but instead of 12 ml of TAS only 10 ml were used. Having been washed, the organic phase in the 350-ml separating funnel was placed in a 250-ml round-bottomed flask

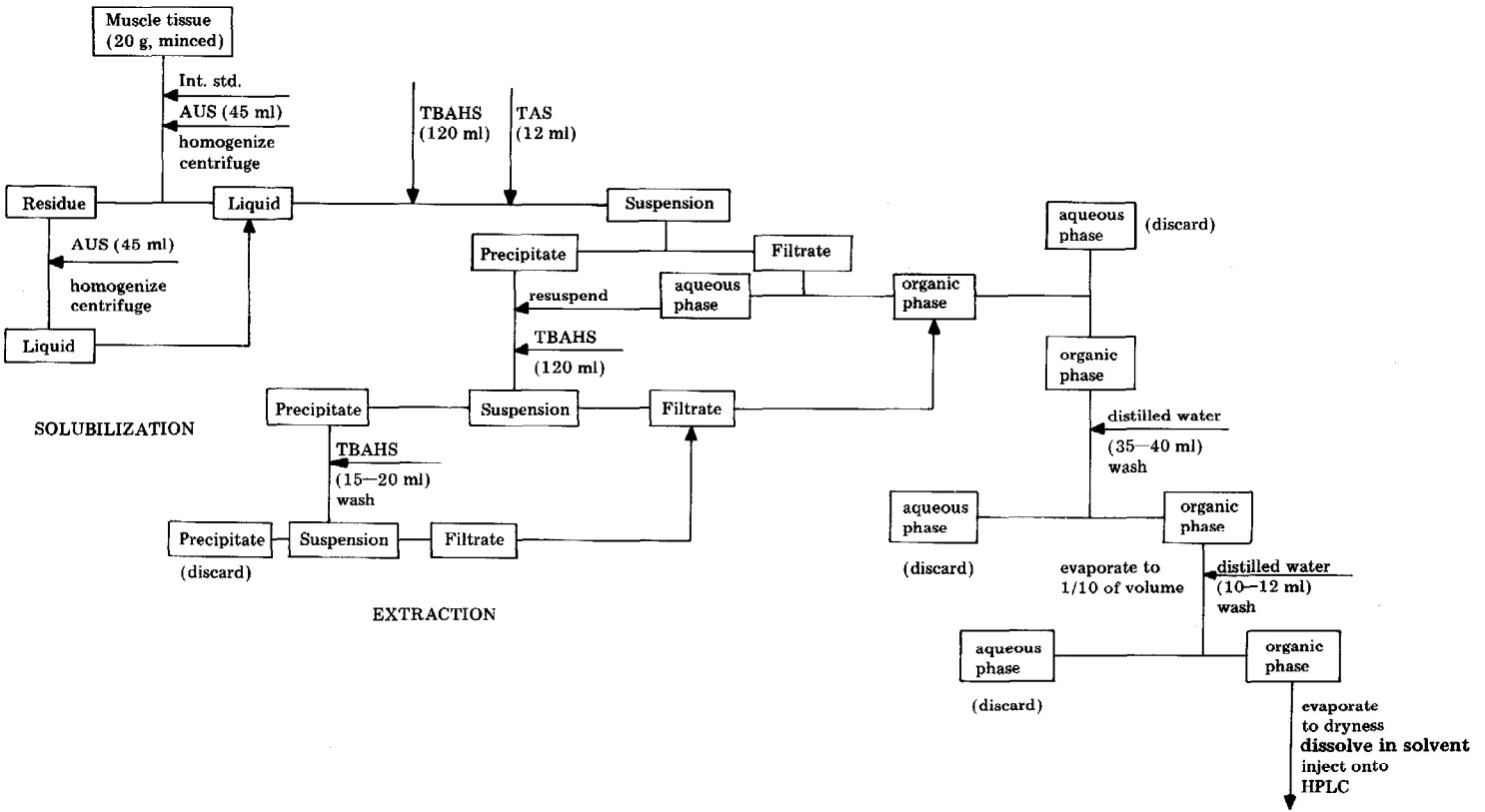

Fig. 1. Flow chart for the isolation procedure (Procedure A).

and the brown solution was evaporated to dryness. The residue in the flask was quantitatively transferred to a 100-ml separating funnel, using about 25—30 ml of hexane and two 10-ml portions of BBS. The contents of the separating funnel were thoroughly shaken and, after separation, the lower phase was collected in a small beaker; the organic phase was discarded. The lower phase was replaced into the separating funnel and the extraction of the pigments from it was repeated twice with 25-ml portions of hexane. The organic phase portions were discarded.

Next, the pH of the lower phase was adjusted to 4.5 ± 0.2 with concentrated hydrochloric acid. The solution was then transferred to a separating funnel and extracted twice with 40-ml portions of TBAHS. The combined organic phase was placed into a separating funnel and thoroughly shaken with 30 ml of distilled water. The resulting emulsion was centrifuged, then the organic phase was drawn off, placed in a 50-ml round-bottomed flask and its volume was reduced to 10 ml on a rotating evaporator. Using some ethyl acetate, the contents of the flask were carefully transferred to the separating funnel and shaken again with 10 ml of distilled water. The emulsion was centrifuged and the organic phase was drawn off, placed in a small round-bottomed flask and evaporated to dryness.

The dry residue obtained in procedure A or procedure B was redissolved, using about two 0.3-ml portions of chloroform—acetonitrile—methanol (15:15:5) and four small portions of *n*-hexane—chloroform—acetonitrile—methanol (80:12:7:1). The portions were collected in a previously calibrated, glass-stoppered test tube to make a total volume of 2.00 ml. Of the homogeneous solution 5—40 μl were injected onto the column.

For calculations, calibration graphs were prepared in such a way that sulfonamide-free muscle and liver samples were used for procedures A and B, respectively, but solutions A and B also contained increasing quantities of Sq, in series, in addition to the fixed amount of Sd. Peak height ratios (Sq/Sd) were plotted against the Sq concentration in the respective tissue.

HPLC conditions

Analyses were performed with a Varian 8500 pump equipped with a stop-flow septumless injector (Varian, Palo Alto, CA, U.S.A.) and an ISCO Model UA-5 (Type 6) absorbance monitor (254 nm, with peak separator and built-in recorder; Instrumentation Specialties Company, Lincoln, NE, U.S.A.). Separations were effected on a Spherisorb S10 W (10 μm), 250 × 3 mm I.D. column (Chrompack Nederland B.V., Middelburg, The Netherlands). The eluent was *n*-hexane—*n*-hexane (water saturated)—chloroform—acetonitrile—methanol—25% (w/v) ammonia (36:30:15:14.5:4.5:0.05) for procedure A or (37:30:15:14.5:3.5:0.05) for procedure B.

RESULTS AND DISCUSSION

Optimization of the extraction procedure

Although HPLC with UV detection offers good possibilities for the sensitive determination of sulfonamides (as most of these drugs exhibit strong UV

absorption between 240 and 270 nm), trace analysis of sulfa drugs in complex biological matrices also requires highly efficient clean-up techniques.

The amphoteric nature of sulfonamides is well established. At a pH below 2.8 the dominant form of Sq is cationic, while at a pH higher than 6.2 its preferred state is anionic, as shown in Fig. 2. The non-ionic form is dominant between pH 3.5 and 4.5, which is the optimal pH range for simple organic extractions of Sq (see Fig. 3).

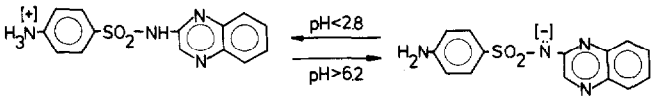

Fig. 2. The amphoteric nature of sulfaquinoxaline.

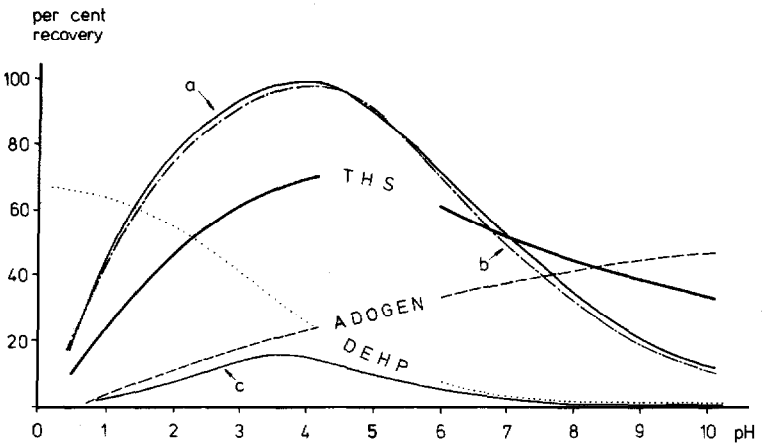

Fig. 3. The efficiency of the extraction of Sq from an aqueous solution (a), urine (b), and an aqueous solution mixed with pulped muscle tissue (c), as a function of pH. For the extraction conditions see Optimization of the extraction procedure. DEHP, ADOGEN and THS curves show the efficiency of the extraction method (average efficiencies from solubilized muscle) involving the use of the respective ion-pairing agent, as a function of pH. Twenty grams of tissue containing a known amount of Sq (in the 0.01—0.10 ppm range) were solubilized by alkaline urea as described in procedure A. The liquid was extracted with 120 ml of ethyl methyl ketone—ethyl acetate (1:1) containing the respective ion-pairing agent in a concentration of 1.5×10^{-3} *M*. The pH of the mixture was adjusted with TAS. Concentrations were all determined by the HPLC method for procedure A (see HPLC conditions).

First, we investigated the extraction of Sq from various aqueous media, using organic solvents only. From a number of solvents and solvent mixtures ethyl methyl ketone—ethyl acetate (1:1) was chosen for the extraction of Sq (and ion-pairs formed with Sq). Fig. 3 shows the efficiency of the extraction (percentage recovery) from a simple aqueous solution, urine and an aqueous solution mixed with pulped muscle tissue, as a function of pH (curves a, b and c, respectively). For curves a and b the concentration of Sq in the aqueous solution was 50 ng/ml and the volume ratio was 1:1. For curve c, 10-ml portions of an aqueous solution of Sq (100 ng/ml) were, after pH ad-

justment, thoroughly mixed with 20-g portions of pulped muscle tissue. The mixtures were allowed to stand for 10 min. The volume of the organic extractant was 100 ml. The extractions were all carried out by shaking the mixtures at about 24°C for 15 min.

As shown, the efficiency of the extraction of Sq from a simple aqueous solution and urine is roughly the same, and at a pH of about 4 it is very high. However, if the aqueous medium also contains a large amount of tissue the efficiency drops considerably, obviously because Sq is strongly bound to tissue proteins (which may account for the poor recoveries obtained at this level with earlier extraction methods).

Next, we attempted to improve the efficiency of the extraction by treating the tissue with 8 *M* urea. Urea is known to be a good protein denaturant, which solubilizes tissue proteins lowering the free energy of the denatured state relative to that of the native structure [13]. Consequently, denaturation by urea should reduce the ability of proteins to bind Sq and improve the recovery of the drug. Although it did, further improvement was needed and was achieved after combining alkaline urea treatment with the technique of ion pairing [14].

Cationic and anionic species (Adogen 464, THS and DEHP, SLS) were tested as counter-ions in a concentration range of 1×10^{-3} to 5×10^{-3} *M*. The efficiency of the ion-pair extraction with three of the counter-ions, as a function of pH, is shown in Fig. 3. As can be seen, the best recovery from tissue was achieved using the cationic counter-ion, THS (1.5×10^{-3} *M*) at a pH of 4.1—4.2. The shape of the THS curve at pH values higher than 6 can be explained by assuming a mixed (non-ionic and ion-pair) extraction mechanism. In the pH range 4.3—6.0 extraction was impossible due to the gel-like structure formed by the proteins precipitated.

Recovery was further improved slightly by the addition of a good adduct-forming agent, isoamyl alcohol, to the organic phase. Thus, the overall recovery with two replicate extractions was 85 ± 8% from muscle and 77 ± 11.2% from liver (average values in the 0.01—0.1 ppm Sq concentration range, see also Table II). Lower recovery data with liver are due to the need for a more laborious extraction procedure aimed at removing tissue pigments from the organic phase.

In the search for a suitable internal standard procedures A and B were also used for the extraction of tissues spiked with other sulfa drugs (Sd, Smd, Sdd, Sgd). The extraction properties of Sd were identical with those of Sq.

Optimization of HPLC parameters

When optimizing chromatographic parameters from the point of view of the lowest possible detection limits, we relied mainly on the work of Karger et al. [15]. Tissue extracts were chromatographed in a number of reversed-phase and normal-phase systems. In each case, naturally occurring and potentially interfering components of tissue made it difficult to optimize separation in such a way that the capacity factor, k', for Sq was in the optimal range of $0.5 < k' < 2.5$. Background interference was a more serious problem with reversed-phase systems. A comparison of chromatograms A and B in Fig. 4 demonstrates the difficulty in achieving a reversed-phase separa-

tion of Sq as well as an additional sulfa drug (to be used as internal standard) from interfering tissue components, if k'_{Sq} is to be kept low.

Figs. 5 and 6 present normal-phase chromatograms obtained on the analysis of tissue samples from control animals (Figs. 5A and 6A) and those obtained from feed-medicated ones (Figs. 5B and 6B). Based on its extraction and retention behaviour Sd was chosen as internal standard. As is shown, interference was eliminated, but, for greater sensitivity, the resolution (R_s) of the compounds of interest was kept to the lowest adequate value.

Following the analysis of 20—30 samples, the column was washed with 50 ml of methanol, then reequilibrated with the eluent. Column performance and calibration graphs were checked by the injection of solutions of Sq and Sd (direct calibration). In the system presented in Fig. 5 (k'_{Sq} = 7; theoretical plate number, N_{Sq} = 4800 per 25 cm, linear velocity, u = 2.5 mm/sec) the detection limit for Sq (twice the noise) was 1.2 ng per injection. With k'_{Sq} at the theoretical optimum, i.e. k' = 1.0, the same system would have permitted a detection limit of 250 pg.

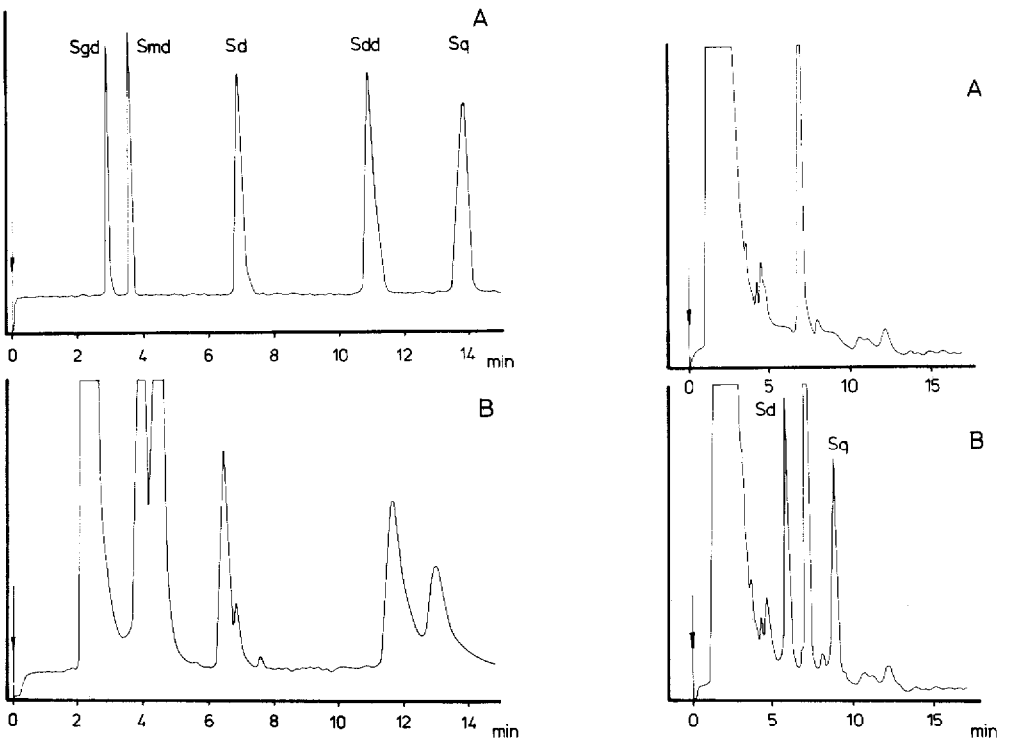

Fig. 4. Reversed-phase chromatography of (A) some sulfonamides and (B) an extract obtained with procedure A from sulfonamide-free muscle tissue (without internal standard). Column, Micro-Pak CH-10 (Varian) (10 μm) 300 × 4.0 mm I.D.; eluent, 0.01 M $(NH_4)_2CO_3$—methanol (90:10); flow-rate, 0.83 ml/min; temperature, ambient.

Fig. 5. Normal-phase chromatography of (A) an extract obtained with procedure A from the muscle tissue of a control animal (without internal standard) and (B) an extract obtained with procedure A from the muscle tissue of a feed-medicated animal. The peaks Sd and Sq represent 50 ng and 37 ng of the respective sulfonamide. The HPLC method for procedure A (see HPLC conditions) was used. The flow-rate was 0.83 ml/min, the temperature was ambient.

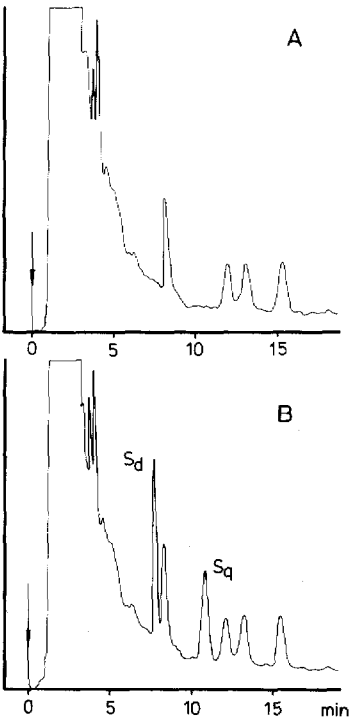

Fig. 6. Normal-phase separations of (A) an extract obtained with procedure B from the liver tissue of a control animal (without internal standard) and (B) an extract obtained with procedure B from the liver tissue of a feed-medicated animal. The HPLC method for procedure B (see HPLC conditions) was used. The flow-rate was 0.83 ml/min, the temperature was ambient.

Linearity, precision and accuracy

No interference originating from diaveridine was noted since the extraction and UV properties of this drug are quite different from those of Sq. The best signal-to-noise ratio was obtained with a fixed-wavelength UV detector. (Sq exhibits an absorption maximum at 252 nm in the eluent.) Linearity of the calibration graphs in the 10 ppb to 10 ppm range was good and fulfilled the criterion suggested by Fowlis and Scott [16].

Precision and accuracy studies were performed at three different concentrations of Sq in spiked control tissues. The results are tabulated in Table I. Coefficients of variation (C.V.) indicate the within-run precision of the quantitative results (samples from the same portion of tissue, on the same day).

Table II summarizes the results obtained on Sq-depletion patterns. Depletion was rapid, especially from liver, during the first two days of withdrawal. A withdrawal period of seven days was found safe enough for achieving no-effect Sq levels in the tissues examined. C.V. values incorporate day-to-day as well as inter-assay (samples originating from different animals) variability.

TABLE I

ACCURACY AND PRECISION OF THE HPLC ASSAY OF Sq IN TISSUES
$n = 8$.

Tissue	Amount added (ng Sq per g tissue)	Amount found (ng Sq per g tissue; mean ± S.D.)	C.V. (%)
Muscle	20	19.2 ± 2.02	10.5
	200	204 ± 11.85	5.8
	1000	988 ± 34.60	3.5
Liver	20	19.3 ± 2.61	13.5
	200	196 ± 14.10	7.2
	1000	1010 ± 49.50	4.9

TABLE II

DEPLETION OF Sq FROM MUSCLE AND LIVER DURING THE WITHDRAWAL PERIOD

Hours of withdrawal (post-treatment)	Sq content (ng/g tissue)							
	Muscle				Liver			
	ng found*			Mean C.V. (%)	ng found*			Mean C.V. (%)
0	956	1070	1010	1010	2210	2150	1750	2015
	1045	980	1050		1900	1830	2320	
	995	975	1010	3.80	2030	2140	1810	10.00
24	631	710	692	668	1010	896	1070	958
	697	628	705		945	1042	937	
	654	683	615	5.50	897	955	870	7.20
48	485	490	505	461	436	412	417	452
	422	418	440		488	501	407	
	462	454	478	6.64	442	465	503	8.46
96	218	251	229	223	108	120	97	99
	240	207	234		96	85	88	
	203	212	209	7.50	95	102	100	10.60
144	76	79	64	69	32	35	25	29
	63	62	74		27	26	31	
	71	70	63	9.22	28	30	27	11.10
168	10	6	7	8	8	10	6	9
	8	9	10		12	8	9	
	6	8	6	15.40	9	7	10	20.00
192	Below the detection limit				Below the detection limit			

*Triplicate samples per chicken.

CONCLUSIONS

The determination of no-effect sulfonamide levels in edible animal tissues poses a complex trace analytical problem due to the fact that sulfa drugs are

strongly bound to tissue proteins. Solubilization of tissues by 8 *M* alkaline urea was found to be effective in improving the efficiency of the trace enrichment procedure. Best recoveries from the solubilized tissues were obtained by the application of an extraction method of mixed (ionic and non-ionic) mechanism.

In the optimization of HPLC parameters from the viewpoint of sensitivity, a compromise had to be struck. In a sufficiently selective normal-phase system (see Fig. 5) the attainable detection limit for Sq was 1.2 ng per injection, almost five times the theoretical value.

The procedures as described above provide reproducible and quantitative methods for the determination of trace amounts of Sq in animal tissues.

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