Supercritical Fluid Extraction of Sulphamethazine and its Metabolites from Meat Tissues

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Abstract

An investigation is reported of factors affecting the supercritical fluid extraction of sulphamethazine and five of its metabolites from spiked meat (swine liver and kidney). The addition of the polar modifier methanol to the carbon dioxide extracting fluid was found to generally enhance recoveries under subcritical and supercritical conditions. Recoveries of the ionic metabolites were increased by up to 72% when employing tetramethylammonium hydroxide for ion pairing in situ with the supercritical fluid extraction. Extraction efficiency is demonstrated to be dependent on the matrix. Extractions of the less polar compounds from the kidney are more successful than from the liver, which corresponds to their partitioning into the supercritical fluid and/or the greater fraction of highly extractable fatty materials. The kidney was more retentive than liver for the relatively more polar compounds, which suggests that the liver offers a less polar environment under the same extraction conditions.

Introduction

Sulphonamides are a class of synthetic compounds that have found widespread use as drugs in veterinary medicine to treat diseases caused by bacterial infection. The problem with using these chemicals lies in their toxicity and carcinogenic properties. For these reasons, they have been replaced largely, but not entirely, by more congenial antibiotics. Routine determinations for sulphonamides in animal tissues and fluids are still necessary to understand the pharmokinetics and toxicological effects. More importantly, there is a need to survey drug residue levels in major food-producing animals (such as cattle, pigs, and sheep) to ensure that there is no risk of secondary exposure to the consumer.

The main difficulty in applying most methods to the determination of drugs in biological samples has been the excessive and variable background interference from co-extracted endogenous material. An additional extraction procedure may be required in such cases to eliminate interferences, especially if high sensitivity is required. This can make the sample cleanup procedure more laborious. Another consideration in many sulphonamide determinations is the need for precise control of pH. Current extraction methods, although sensitive and time-effective, often involve the use of hazardous chemicals. Increasing pressure from regulatory authorities has urged the development of methods that consume less organic solvents. For these reasons, newer and potentially more attractive procedures that employ supercritical fluids are of interest.

Quantitative (ppm) investigations involving supercritical fluids include the extraction of sulphonamides from animal tissues (1–3), whole milk (4), and an aqueous drug formulation (5). A number of studies have shown that the more polar sulphonamides were more difficult to extract and required more rigorous conditions, such as higher methanol modifier concentrations (3) in the bulk carbon dioxide extracting fluid or higher working pressures. More recently, Tena et al. (6) reported the employment of ion-pairing reagents to extract ionic sulphonamides from solid supports.

Parks et al. (3) found that liver and kidney, the important organs of metabolism and excretion, yield lower sulphonamide recoveries with supercritical carbon-dioxide-based fluids than muscle tissue does. This is probably a consequence of the less fibrous content of liver and kidney.

Many of the studies with supercritical fluids have concentrated on the analysis of the parent sulphamethazine, which, although polar, has more polar and ionic metabolites that are equally, or more so, of interest. In this study, we investigate quantitatively the supercritical fluid extraction (SFE) of some of the polar metabolites (Figure 1) from spiked swine liver and kidney tissues using methanol-modified carbon dioxide. The extraction of sulphamethazine is also included. The effect of in situ ion-pairing reagents is examined for the extraction of ionic sulphamethazine metabolites.

Experimental

Materials and chemicals

Sulphamethazine (SMZ) was purchased from Aldrich (Milwaukee, WI), and its derivatives, N^4-acetylsulphamethazine

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(AC), desaminosulphamethazine (DES), N^4-glucosylsulphamethazine (GS), N^4-glucuronylsulphamethazine (GR), and N^4-sulphatosulphamethazine (SO_4), were synthesized in the laboratory (7). A solution of these sulphonamides was made up in methanol (HPLC grade)(Fisons, Loughborough, UK). The concentration of each compound in this standard was known; the values were around 0.2 mg/mL in the standard used in extraction from the "inert" matrices and 2.5 mg/mL in the standard used for the tissue samples. Hydromatrix was obtained from Varian (Harbor City, CA). The swine tissues were from a local slaughterhouse where it was known that the animals had not previously been exposed to veterinary drugs. For ion-pairing experiments, the reagents tetrabutylammonium bromide (TBAB) (Fluka Chemicals Ltd., Gillingham, UK) and tetramethylammonium hydroxide (TMA) (25% wt solution in methanol containing 5–10% water)(Aldrich) were used. The tetrabutylammonium bromide was made up as a solution in water (0.6 g/mL).

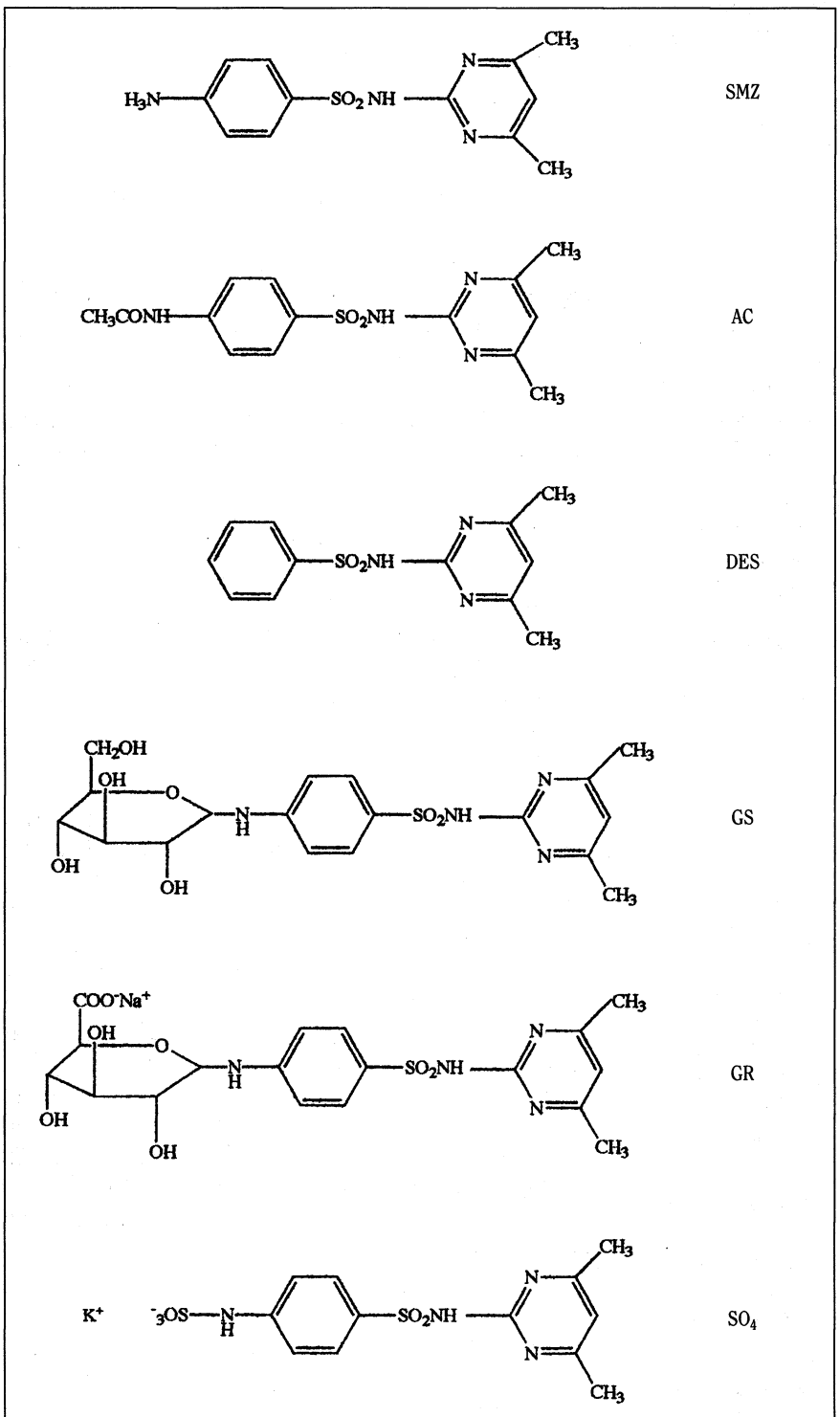

Figure 1. Chemical structures of sulphonamides used in this study.

Sample preparation

The wet liver and kidney tissues were macerated into a smooth paste with a domestic food blender. The support material Hydromatrix was homogeneously premixed with the respective wet tissues in a 1:1 (wt/wt) ratio before spiking. Freeze-dried tissues were ground into a uniform powdered state. Extractions with wet and ground freeze-dried tissues involved spiking at 50-ppm and 100-ppm levels, respectively.

Filter paper extractions were prepared by applying a 100-µL spike of standard to a fluted strip. This was then allowed to dry either naturally or in a gentle stream of warm air. For all other extractions, the sample was loaded into the SFE cell and spiked along the longitudinal central axis. Ion-pairing reagents (50–100 µL) were introduced into the cell in the same way. Some time (~15 min) was allowed for the methanol to evaporate before the cell was closed and incorporated into the SFE system.

Supercritical fluid extraction

Extractions were performed using an Isco SFX2-10 extractor (Isco, Lincoln, NE). This system comprised two syringe pumps: one to supply liquefied carbon dioxide and the other to supply methanol modifier. Mole percentages were calculated from the volume percentages of carbon dioxide and methanol that were delivered. Liquefied carbon dioxide was maintained by recirculating a cooling fluid mixture (polyethylene glycol and water at about –10°C) around the carbon dioxide pump head. Mixing of the two fluids occurred at a common tee-piece. The homogeneous fluid was then delivered to the extraction cell, housed within a temperature-controlled oven. The temperature of the oven was maintained above the critical temperature of the fluid, which was calculated using SFSolver software (8). Static extractions were performed at 400 atm when ion-pairing reagents were used by closing the in-line valve immediately after the extraction cell. A time period of 30 min was allowed for equilibration

of the spike and ion-pairing reagent. Following this, the valve was opened, and the extraction was allowed to proceed dynamically. The cell was kept pressurized (at 400 atm) during this purging process by using a piece (10–15 cm in length) of linear deactivated fused silica capillary tubing (15–30-µm i.d.)(Composite Metal Services Ltd., Harrow, UK) as a restrictor. For the extraction of "real" samples, it was sometimes necessary to heat this restrictor in a stream of warm air to prevent blockage.

All extracts were collected using two vials in series. The restrictor was purged into the first septum-sealed vial, which contained 3–10 mL of methanol. The expanded carbon dioxide gas was then vented through a piece of Teflon tubing into the second vial, which contained methanol. The solutions were combined, centrifuged, or cleaned up as necessary and then blown down to dryness under a gentle stream of nitrogen (BOC Ltd., Guildford, UK) at room temperature. The cleanup step was necessary when there was a substantial amount of co-extracted endogenous material. This was affected by in-vial partitioning using chloroform (0.5 mL) and acetonitrile–buffer (0.5 mL)(the mobile phase used for HPLC analysis). Two clear layers formed, divided by a band of solid matter. The buffer layer was carefully removed using a syringe and evaporated under nitrogen to obtain the final extract. The extracts were dissolved in an appropriate amount of methanol (100–750 µL), syringe filtered (2-µm pore size), and then subjected to HPLC analysis.

Table I. Effect of Methanol-Modifier Concentration on the Extent of Extraction (from Filter Paper) for a Range of Sulphonamides

Mol % methanol	% Recovery*					
	GS	GR	SO_4	SMZ	AC	DES
Supercritical extractions						
30-min extractions						
0.0	0	0	0	11	3	22
0.1	0	0	0	31	11	35
0.2	0	0	0	31	17	33
0.5	0	0	0	50	15	38
60-min extractions						
0.1	5	0	0	76	77	87
0.2	11	0	0	81	91	94
Subcritical extraction						
30-min extraction						
0.5	37	4	15	21	40	38

* Mean recovery (n = 3), RSD = 2–19%. Pressure, 400 atm; average liquid flow rate, 0.8 mL/min. Supercritical fluid extractions with 0, 0.1, 0.2, and 0.5 mol % methanol are at 40, 55, 75, and 140°C, respectively. The subcritical extraction is at 40°C.

Table II. Comparison of Extraction Recoveries from Hydromatrix Using In Situ Ion-Pairing SFE

	Ion-pairing methanol	% Recovery*					
		GS	GR	SO_4	SMZ	AC	DES
1	×	79	0	0	97	90	102
2†	(a)√TBAB	86	0	0	115	91	87
	(b)√TBAB	78	12	81	128	80	79
3	√TMA	75	29	83	131	84	79

* Mean recovery (n = 3), RSD = 7–16%.
Conditions: 0.1 mol % methanol in carbon dioxide at 400 atm and 60°C; average liquid flow rate, 0.6 mL/min.
√ = 30 min static extraction followed by 2-h dynamic.
× = Not used
† (a) The TBAB is present in its crystalline form mixed with Hydromatrix; (b) the TBAB is injected as an aqueous solution with the standard sulphonamide spike injection.

HPLC analysis

A reversed-phase ion-pairing HPLC system was used to analyze the extracts. A C_{18} column was used (25 cm × 4.6 mm, Spherisorb)(Phase Separations, Deeside, UK) with a C_{18} precolumn (2 cm). The system was equipped with a multisolvent delivery system and controller to provide the 15:85 acetonitrile–aqueous acetate (50 mM) buffer solution mobile phase. This was maintained at a pH of 5.0. The buffer also contained the ion-pairing reagent TBAB (10 mM). The flow was controlled at 1.0 mL/min for the first 17 min of the run and then 1.2 mL/min for the remainder. An autoinjector (equipped with a 10-µL sample loop) was used to introduce the sample. Analytes were detected by ultraviolet (UV) detection at 266 nm. Quantitation was carried out by calculation of peak areas and comparison with the standard.

Results and Discussion

Optimizing conditions for SFE

The optimum temperature for the extraction of the sulphonamides used in this study from Hydromatrix and filter paper

Table III. Extraction Recoveries from Wet and Ground Freeze-Dried Swine Liver and Kidney Samples

	TMA	% Recovery*					
		GS	GR	SO_4	SMZ	AC	DES
Wet liver	×	73	0	41	70	72	91
	√	61	10	65	53	50	62
Wet kidney	×	24	0	0	97	93	97
	√	16	0	0	65	42	63
Ground freeze-dried liver	×	36	0	0	6	54	77
	√	20	0	0	9	42	53
Ground freeze-dried kidney	×	8	0	0	26	72	82
	√	47	23	72	61	58	65

* Mean recovery (n = 2) (calculated at similar flow rates), RSD = 2–24%.
Conditions: 0.1 mol % methanol in carbon dioxide at 400 atm and 60°C; average liquid flow rate, 0.8 mL/min.
TMA extractions: 30-min static, 2-h dynamic; all other extractions: 2-h dynamic.
√ = Present
× = Not present

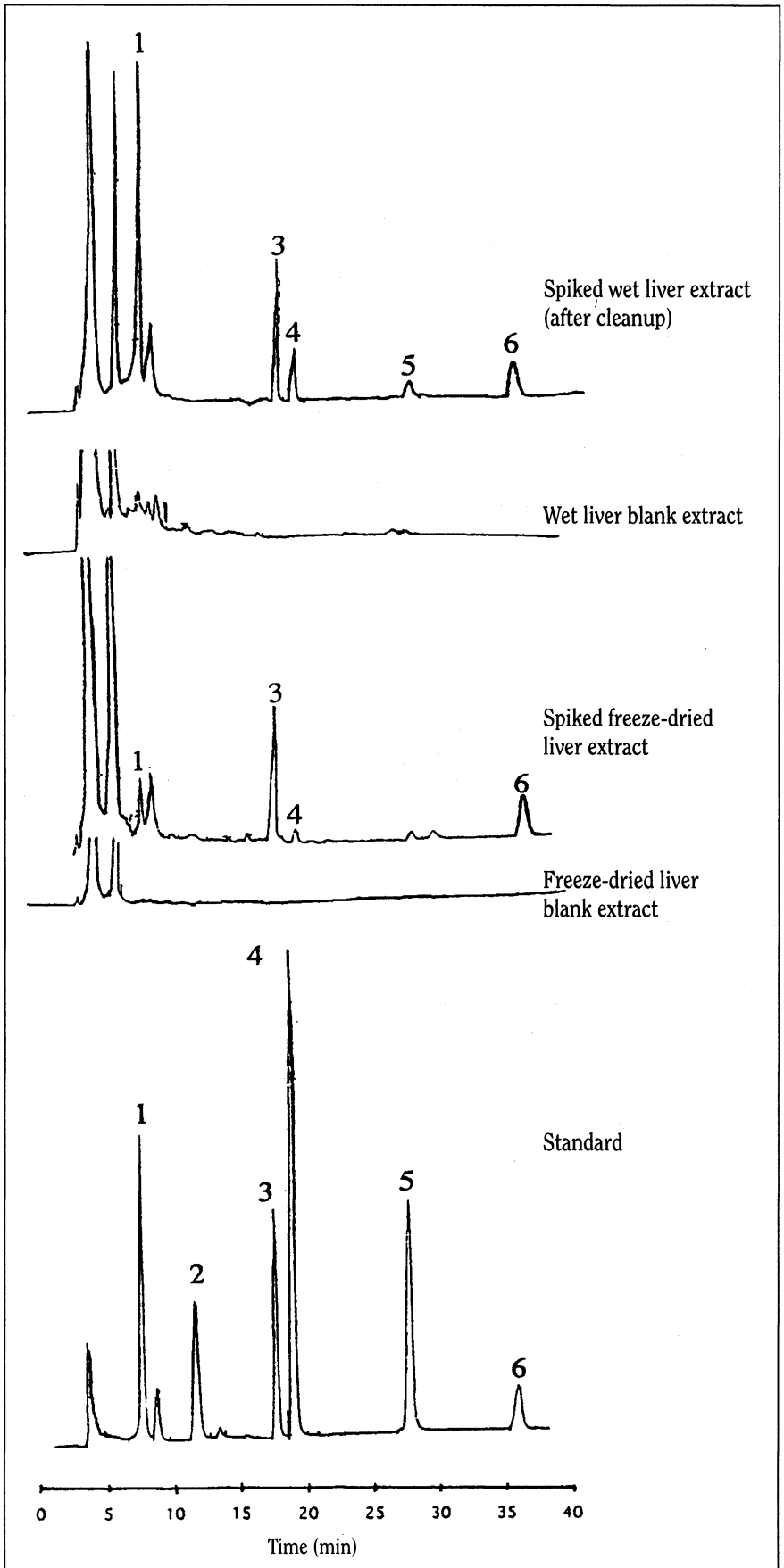

Figure 2. Typical HPLC chromatograms of swine liver extracts. Peaks: 1, GS; 2, GR; 3, AC; 4, SMZ; 5, SO_4; 6, DES.

with 10% methanol-modified carbon dioxide was found to be 60°C at 400 atm. Dynamic extractions were performed by maintaining a continuous flow of the extraction effluent. At the flow rates used, two-vial collection was found to be most effective. These conditions were used for all further experiments. The very polar and ionic metabolites (GS, GR, and SO_4) could not be detected in the SFE effluent after 30 min (Table I); however, after a 60-min extraction, they were detected, which illustrates their low solubility in the supercritical fluid. Using a greater mole percentage of methanol in an overall subcritical fluid showed the expected enhanced solubility of the more polar metabolites (Table I).

Increasing the modifier concentration further and working at either subcritical or supercritical conditions is one method of increasing recoveries (Table I), but this method nullifies the aim of reducing organic solvents. Another alternative is to increase the density of the SFE phase.

However, with most SFE systems, high pressures (in excess of 600 atm) are not readily attainable, and special extraction cells along with high pressure valves and fittings must be used. Derivatization of GS and SO_4 is impractical because the parent sulphamethazine is produced (7), which could not then be independently quantitated. Ion pairing seems to be a more acceptable approach for the least soluble, ionic sulphonamides. In effect, the cation (Na^+ or K^+, depending on the compound of interest) of the ionic sulpha compound exchanges with the ion-pairing reagent (either $[CH_3]_4N^+$ or $[C_4H_9]_4N^+$). The polarity of the resultant GS and SO_4 ion-pair products should be reduced from that of the initial, and their solubility should be enhanced.

Although a number of studies have described in situ SFE ion pairing, to our knowledge, the technique has not been applied to animal tissues until now.

SFE from solid supports using in situ ion-pairing reagents

Assuming a perfectly permeable sample, the ideal scenario would involve initial saturation of the extracting phase with the ion-pairing reagent. This is to ensure that the reagent is accessible to the analytes and to the relevant reactive sites. For complex matrices such as tissues, direct exposure of the analyte(s) to the ion-pairing reagent is not easily achievable; numerous chemicals (matrix components), interactions, physical

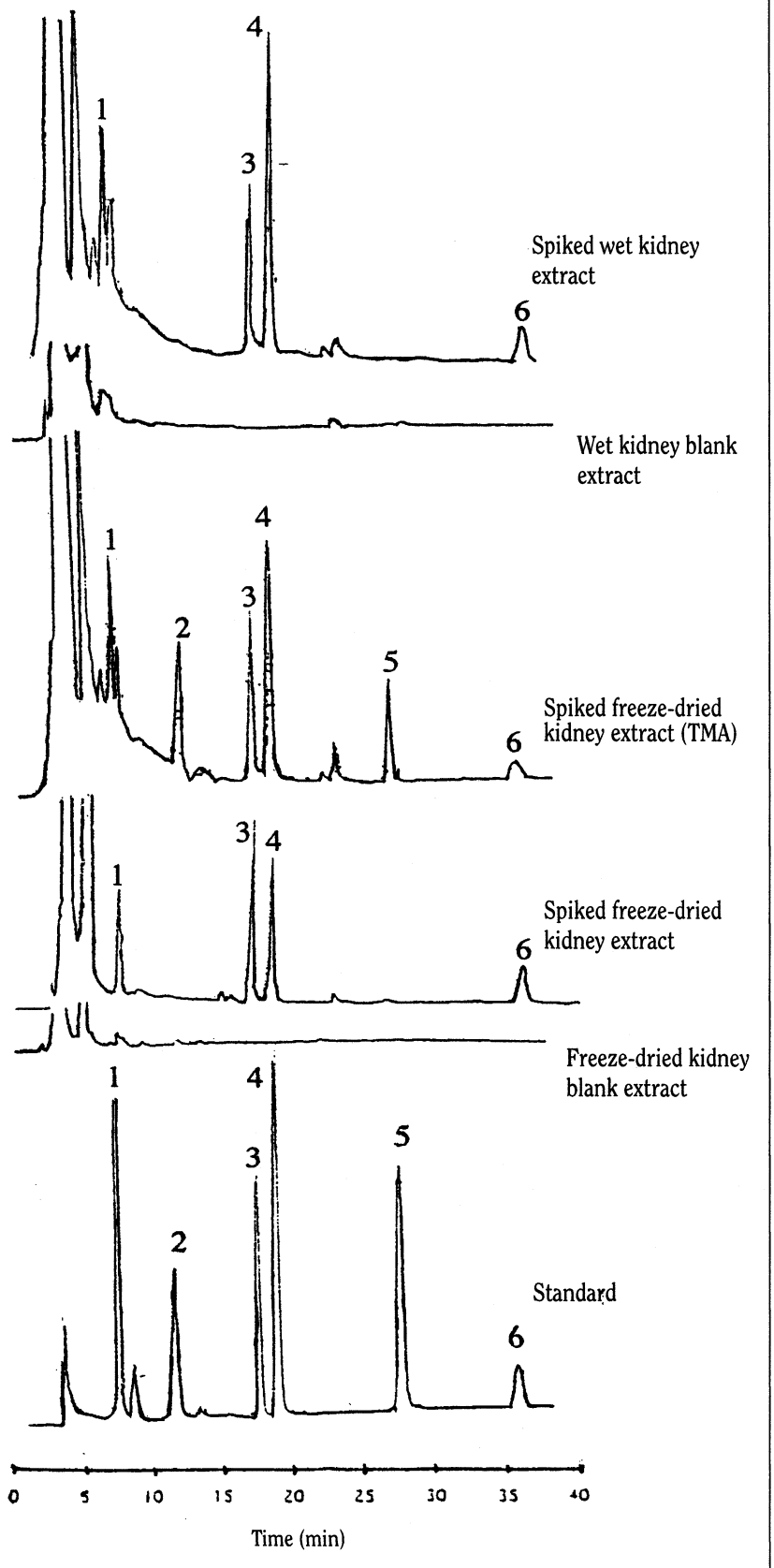

Figure 3. Typical HPLC chromatograms of swine kidney extracts. Peaks: 1, GS; 2, GR; 3, AC; 4, SMZ; 5, SO_4; 6, DES.

limitations (sample morphology, for example), and molecular configurations determine the success. Hydromatrix was found to give comparable recoveries to filter paper for the three less polar compounds and much better recoveries for the GS, which suggested a less severe matrix interaction. For these reasons, experimental measurements of the effectiveness of the ion-pairing reagents TBAB and TMA were conducted by applying the technique first on Hydromatrix. A 2-hr period was used for exhaustive (dynamic) extraction. The results of the spiking experiments are shown in Table II.

The use of TMA (available in a methanol solution) as an ion-pairing reagent in SFE is known; Field et al. (9) have used it for sulphonate compounds. The effect of TBAB in HPLC analysis of the sulphonamides used in this study is known; its use in SFE, however, is expected to be limited (by its insolubility in methanol) for the complex tissue matrix form used in this study. (Direct contact of TBAB with an aqueous-based liquid matrix would be more appropriate because TBAB is water-soluble.) As a point of interest, the reagent was used to examine if its presence had any effect on the extraction.

The presence of either the TBAB or TMA ion-pairing reagents has about an equal effectiveness in aiding the extraction of SO_4 (Table II). Both reagents brought about increased extraction yields of GR, but to a lesser degree; TMA was superior. A comparison of the recoveries (2a and 2b in Table II) emphasizes the importance of contact between the ion-pairing reagent and the drug compounds.

Effect of water and pH

In some systems, water is introduced with the ion-pairing reagent. Under the experimental conditions used in this study, carbon dioxide dissolves in water (10); some of it reacts to produce carbonic acid. This reaction brings the pH of the mobile phase to at least 5. A continual supply of fresh carbon dioxide overcomes any buffer action in the sample. The acidic conditions explain why recoveries of the GR metabolite are low, whereas those of SMZ are unrealistically high (115, 128, and 131%); some of the glucuronyl metabolite hydrolyzes to give the parent sulphamethazine (7). This effect in tissue systems, which contain large amounts of water (up to 70–80%, w/w), would then seem unavoidable. If the pH of the supercritical extractant were known, it would be possible to calculate

the ionization constants for the sulphonamides. This would give some indication of their relative stability in the medium. The effect of differing pH conditions on the SFE of sulphonamides has been investigated by Tena et al. (6).

SFE from meat tissues using in situ ion-pairing reagents

Generally, the SFE of wet biological samples is difficult. In this study, 1:1 mixtures of the "wet" tissue supported on Hydromatrix were extracted. For comparison, the ground freeze-dried tissues were also extracted. Samples were fortified at parts-per-million levels. The results are shown in Table III.

Variations between results of similar experiments were the combined result of precipitation of extractable materials and water condensation in the restrictor. Any flow problems due to these effects were quickly remedied by heating the restrictor in a warm stream of air. The use of the ion-pairing reagent (TMA) affected the trapping procedure, as shown by lower recoveries of the less polar compounds. Despite this, the data obtained from the experiments displayed some clear trends.

It is well-known that the solubility of fats (i.e., lipids, triglycerides, etc.) in supercritical carbon dioxide is high (11). In fact, this is often a problem in the SFE of low to medium polarity drugs from biological samples. In this study, visual comparison of extract solutions showed a greater content of suspended co-extracted endogenous matter (mainly fatty material) from the kidney than from the liver. Cleanup was easily achieved by either centrifuging the extracts or by simple liquid partitioning of fats. The HPLC chromatograms of typical liver and kidney extracts (Figures 2 and 3, respectively) were adequately free of interferences.

Although the results of extraction experiments with spikes cannot accurately predict behavior from naturally contaminated samples, they do offer a basis for establishing suitable conditions and are an indication of limiting factors. Overall, the data in Table III illustrate consistently higher recoveries of the relatively less polar compounds from the kidney rather than from the liver. This trend is a consequence of the less polar metabolites and SMZ preferentially partitioning into the supercritical fluid and the highly extractable fatty materials, which confirms the hypothesis of Hedrick et al. (4). The more polar glucosyl metabolite (GS) and ionic compounds were more strongly retained by the kidney than the liver in the absence of the ion-pairing reagent. These observations suggest that the liver sample offers a less polar environment than the kidney under the same extraction conditions.

The nature of analyte–matrix interactions clearly changes with freeze-drying. Lower recoveries of sulphonamides result from freeze-dried tissues in comparison with "wet" tissues, which suggests stronger specific matrix interactions in freeze-dried tissues. The absence of water results in stronger sulphonamide–matrix binding. This is most noticeable in the results obtained for sulphamethazine. The effect is more pronounced in liver than in kidney.

The addition of the ion-pairing reagent in the extraction of the polar metabolites generally enhanced recoveries in "wet" liver and freeze-dried kidney; the greatest improvement was with the sulphato metabolite, which showed an increase from 0 to 72%. The ineffectiveness of TMA in the other experiments is a likely consequence of poor accessibility of the reagent to the relevant analyte sites.

Conclusion

Ionic and very polar sulphonamides can be extracted from parts-per-million-level spiked swine liver and kidney, using methanol-modified carbon dioxide and in situ ion-pairing. The highest recoveries of the "low" polarity sulphonamides (SMZ, AC, and DES) were achieved with the "wet" kidney. Recoveries ranged from 93 to 97% using 10% methanol–carbon dioxide at 400 atm and 60°C. Using TMA as an ion-pairing reagent increased recoveries of the polar and ionic sulphonamides up to 72% (for SO_4). Overall, the poorest recoveries obtained were for the polar GR, which seemed to be particularly sensitive to acidic environments.

The extraction efficiency of polar analytes is impeded by their retention on the matrix and their poor solubility in the supercritical fluid. The type of matrix is critical to the recovery. Without efficient cleanup, poor peak integration (in the analysis) can be a source of low recovery data. For the purposes of this study, with parts-per-million-level spiked samples, cleanup was quickly achieved by centrifuging or in-vial partitioning. Extractions on samples contaminated at parts-per-billion levels may require improved cleanup methods.

In summary, the extraction of drugs from complex matrices is very complex; numerous influencing factors may limit or enhance the procedure. Because of the complexity of the technique and variabilities between matrix samples, the only way to optimize the SFE procedure is through a series of evolutionary and systematic experimental processes in which each experimental parameter is optimized.

Acknowledgment

We thank the Ministry of Agriculture, Fisheries, and Food for support of this work through the award of a studentship to Nooreen Din.

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Manuscript accepted August 15, 1996.