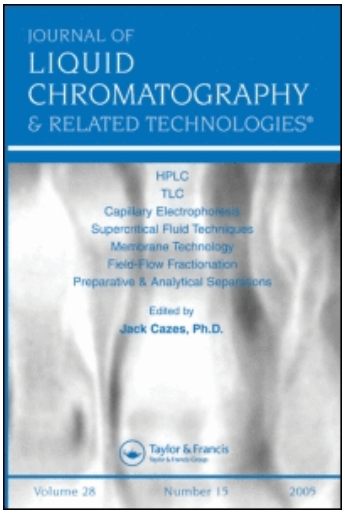

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Determination of Sulfadimethoxine, Sulfamethoxazole, Trimethoprim and Their Main Metabolites in Lung and Edible Tissues from Pigs by Multi-Dimensional Liquid Chromatography

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DETERMINATION OF SULFADIMETHOXINE, SULFAMETHOXAZOLE, TRIMETHOPRIM AND THEIR MAIN METABOLITES IN LUNG AND EDIBLE TISSUES FROM PIGS BY MULTI-DIMENSIONAL LIQUID CHROMATOGRAPHY

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ABSTRACT

A practically fully automated liquid chromatographic method was developed in order to investigate the tissue distribution of sulfadimethoxine, sulfamethoxazole, trimethoprim and their main metabolites in lung tissue and edible tissues from pigs. The sample pre-treatment consisted of a simple extraction with an aqueous buffer solution. The filtered extract was injected onto a liquid chromatographic system which consisted of an on-line gel-

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permeation column, a small preconcentration column and an analytical HPLC column. Parent drugs and metabolites were separated by reversed-phase HPLC and detected by UV absorption. Recoveries of parent compounds and their metabolites in spiked lung, muscle, kidney and liver tissue were constant and varied between tissues from 60-85 %. Detection limits in these tissues were 10-50 ng/g tissue. Application of the method for the analysis of drug residues in tissue samples obtained from treated pigs is described.

INTRODUCTION

Combinations of sulfonamides and trimethoprim are commonly used for the treatment of respiratory and gastro-intestinal tract infections in food-producing animals. The large scale application of, in particular, sulfonamides has led to the occurrence of residues of parent compounds and metabolites in edible tissues of food-producing animals. In order to investigate the tissue distribution of sulfadimethoxine (SDM), sulfamethoxazole (SMX), trimethoprim (TMP) and their main metabolites in lung (*target*) tissue and edible tissues from pigs, a sensitive liquid chromatographic (LC) method has been developed. The determination of SDM, SMX, TMP and their main metabolites in porcine plasma by column switching HPLC has been described previously [1]. The metabolites under investigation were the *N*4-acetyl sulfonamides (AcSDM and AcSMX) and 3'- and 4'-demethyl trimethoprim (also called M4 and M1 respectively).

Multi-dimensional liquid chromatography refers to the technique in which fractions from one column are selectively transferred to one or more secondary columns for further separation [2]. Sometimes this technique is also referred to as coupled column chromatography [3]. In the present study two different LC techniques were combined, namely gel-permeation chromatography (GPC) and reversed-phase chromatography (RPC). On-line combinations of GPC (or size-exclusion chromatography) and RPC were used for the determination of additives, constituents or contaminants in foods and

drugs in plasma [2-8]. However, these methods are not sensitive enough for our purposes since detection limits are > 0.5 μg/g or μg/ml. An elegant way to enhance sensitivity is to introduce the fraction of the gel-permeation eluate containing the analytes onto a small preconcentration column as was described by Kraak *et al.* [9]. Trace enrichment of the selected fraction is achieved and the investigated compounds are introduced onto the analytical HPLC column by desorbing them from the small preconcentration column with the HPLC eluent.

The presented method resembles the on-line combination of dialysis and trace enrichment applied in CF-LC (Continuous Flow-Liquid Chromatography) [10-12]. The determination of residues of sulfonamides by CF-LC was described by Aerts *et al.* [11]. These investigators applied post-column derivatization of sulfonamides with p-dimethylaminobenzaldehyde to enhance both the selectivity and sensitivity. However, derivatization of the *N*4 amino group of the sulfonamides excludes detection of the *N*4-acetyl metabolites. The purpose of this study was to develop a practically fully automated, on-line, LC method for the analysis of residues of veterinary drugs, as well as their main metabolites, in edible tissues. Sensitivity of the method should be below the established maximum residue levels in The Netherlands (i.e. 0.1 μg/g for sulfonamides and 0.05 μg/g for TMP). Application of this method to the analysis of tissue samples obtained from pigs treated with these drugs is described.

MATERIALS AND METHODS

Chemicals and Reagents

Sulfadimethoxine (SDM), sulfamethoxazole (SMX) and trimethoprim (TMP) were obtained from Sigma (St. Louis, MO, USA). *N*4-acetyl

sulfadimethoxine (AcSDM) and *N*4-acetyl sulfamethoxazole (AcSMX) were kindly donated by Dr. T.B. Vree (St. Radboud Hospital, Nijmegen, The Netherlands); 4'-demethyl trimethoprim (M1) and 3'-demethyl trimethoprim (M4) were kindly donated by Dr. M. Holck (Hoffmann-La Roche, Basel, Switzerland).

All chemicals were of analytical grade, triethylamine (TEA) was purchased from Fluka Chemie AG (Buchs, Switzerland) and all other chemicals were obtained from Merck AG (Darmstadt, FRG).

Standards and Spiked Tissue Samples

Spiked tissue was prepared by adding a small volume (≤ 100 μl) of an aqueous standard solution to blank, ground tissue from pigs and left to stand for 30 min at room temperature to allow possible protein binding of the drugs and metabolites before the extraction was carried out. Aliquots of the standard solutions (≤ 100 μl) were also used to spike blank tissue extracts.

Instrumentation and Chromatographic Conditions

The liquid chromatographic (LC) system consisted of three solvent-delivery pumps, model SF-400 (Applied Biosystems, ABI, Maarssen, The Netherlands), two automatic six-port switching valves (Multiport Streamswitch, Spark Holland, Emmen, The Netherlands), a thermostatically controlled column oven (ABI), an UV-VIS detector, model 204 (Linear Instruments Corp., Reno, NV, USA) and an auto-sampling injector, model 231-401, equipped with a cooled sample tray (Gilson Medical Electronics Inc., Middleton, WI, USA). The column switching program was operated by the autosampler. The temperature of the HPLC column was kept at 30°C. The

detection was performed at 270 nm (sulfonamides and their metabolites) or at 240 nm (TMP and its metabolites).

The gel-permeation column was a Progel-TSK column, type G2000 SW, 300 x 7.5 mm I.D. (Supelco Inc., Bellefonte, PA, USA). The applied gel-permeation guard columns were Progel-TSK columns, type SW, 75 x 7.5 mm I.D. (Supelco Inc.). Preconcentration was carried out on a small stainless steel column (10.0 x 3.0 mm I.D.) dry packed with PLRP-S (15-25 μm, 100 Å, Polymer Laboratories, Church Stretton, UK). The analytical columns were a Supelcosil LC-18-DB HPLC column, 250 x 4.6 mm I.D. (Supelco Inc.) and a Chromspher C18 HPLC column, 200 x 3.0 mm I.D. (Chrompack, Middelburg, The Netherlands), both columns were used in combination with reversed-phase guard columns.

The applied eluents for GPC/preconcentration and RPC are given in table 1. All eluents were filtered and degassed with helium before use. The flow rate for the Chromspher C18 column was 0.8 ml/min and for all other columns 1.0 ml/min.

For the extraction of tissue samples a Stomacher laboratory blender, model 400 (Lameris, The Netherlands), was used. Extracts were centrifuged in a cooled centrifuge (Hettich, Mikro Rapid 'K, type 1306, Dépex BV, The Netherlands) and filtered over a disposable filter, 0.45 μm Acrodisc no. 4184 (Gelman Sciences Inc., Ann Arbor, MI, USA).

Sample Pre-treatment

At section of the pigs, tissue samples (100-200g) were collected and immediately frozen at -20°C. After defrosting, visible collagen was removed from the tissue. The sample was homogenized using a blade mixer and divided

into smaller portions of approximately 25g. One portion was analysed in duplicate and the others were stored at -20°C.

The sample pre-treatment for TMP and the sulfonamides was slightly different.

Trimethoprim and metabolites. Ground tissue (10g) was extracted for 3 min. with 20 ml 0.2 M. NaAc pH 5.0 in a Stomacher blender. The homogenate was centrifuged for 10 min. at 10,000g. To 4 ml of supernatant 80 μl of a 5% sodium azide solution was added, the pH of the extract was adjusted to 7.0 with 2.5 M KOH and centrifuged again for 10 min. at 10,000g. The supernatant was filtered (0.45 μm) and 0.5 ml of the filtrate was injected onto the LC system.

Sulfonamides and metabolites. Ground tissue (10g) was extracted for 3 min. with 20 ml 0.2 M. NaAc pH 8.6 in a Stomacher blender. The homogenate was centrifuged for 10 min. at 10,000g. To 4 ml of supernatant 80 μl of a 5% sodium azide solution was added, the pH of the extract was adjusted to 4.6 (SMX and AcSMX) or 6.0 (SDM and AcSDM) with concentrated HAc. Muscle, kidney and liver extracts were partially deproteinized by warming at 37°C for 30 min. This step can be omitted for lung tissue extracts. The extracts were centrifuged for 10 min. at 10,000g; lung, muscle and kidney extracts were centrifuged at room temperature and liver extracts at 5°C. The supernatant was filtered (0.45 μm) and 0.5 ml of the filtrate was injected onto the LC system. Liver extracts were kept at 5°C until analysis.

Column Switching Chromatography

The column switching program can be divided into three steps: GPC, preconcentration (trace enrichment) and RPC. The column switching diagram is given in figure 1A.

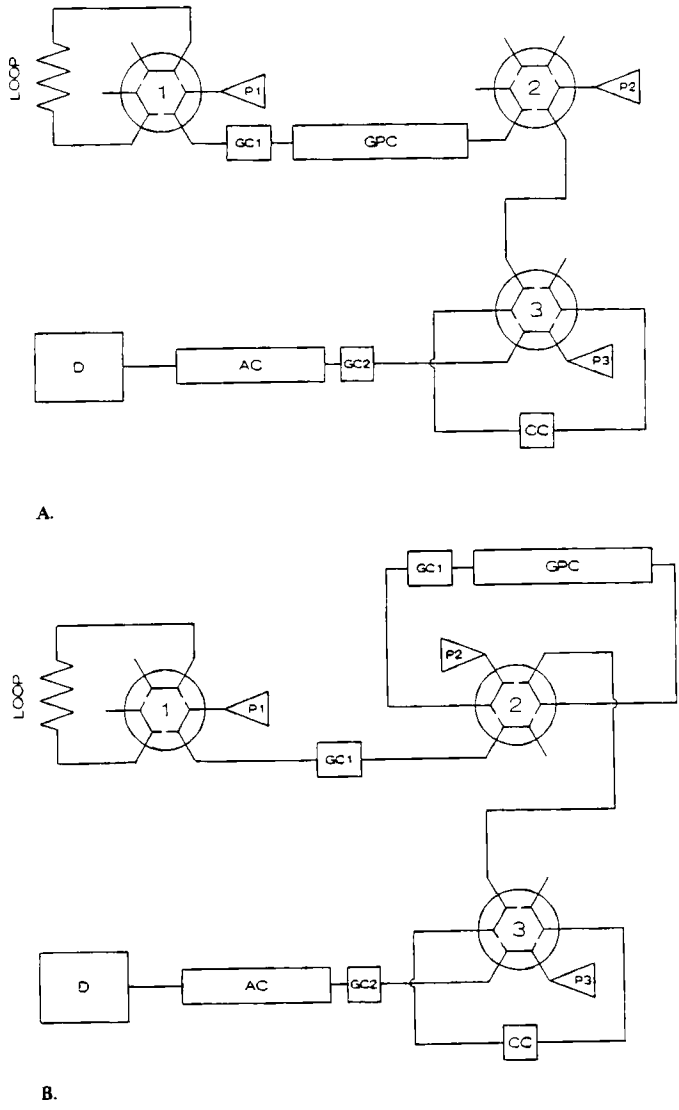

FIGURE 1. Schematic representation of the multi-dimensional liquid chromatographic system, applying one (A) or two (B) gel-permeation guard columns. The following abbreviations were used: P1, P2, and P3: pumps, GC1 and GC2: gel-permeation and reversed-phase guard columns, respectively, GPC: gel-permeation column, CC: (pre)concentration column, AC: analytical column, D: Detector.

First of all the elution behaviour of the analytes on the GPC column was determined with standard solutions and spiked (blank) tissue extracts. The influence of the pH and the molar concentration of the buffer solution on the retention volumes and the resolution were determined. On-line clean-up of the injected aqueous tissue extract was carried out with the GPC column and the fraction of the eluate containing the analytes was introduced onto a small preconcentration column by switching valve 2. In order to check the loss of analyte during preconcentration, different flush volumes (up to 30 ml) were tested. After trace enrichment was completed valve 3 was switched and the analytes were introduced onto the analytical column by desorbing them from the small preconcentration column with the HPLC eluent (in a backflush mode) for 2 minutes. During the separation of the analytes on the HPLC, valves 2 and 3 could be switched back to the original position and another sample could be introduced onto the GPC column.

In order to prolong the life-time of the gel-permeation column it was investigated whether this could be achieved by introducing a second gel-permeation guard column to the described system (Figure 1B).

Quantification

The studied concentration ranges were 0.05-1.0 μg/g tissue for the sulfonamides and their metabolites and 0.025-0.5 μg/g tissue for TMP and its metabolites. With each series of tissue samples three spiked tissue samples were analysed to check linearity and recovery. The coextracted volume of tissue fluid was determined after freeze drying of a random selection of tissue samples. Analyte concentrations were determined by comparison of peak heights.

Recovery

Known amounts of the analytes were added to blank, ground tissues of pigs. The tissue samples were extracted as described and standards were correspondingly diluted, taking the volume of tissue fluid into consideration.

Animal Experiment

Medicated feed, containing 125 mg SMX in combination with 25 mg TMP per kg feed, was given twice daily to 24 healthy pigs during 7 days. After cessation of the feed medication, pigs (n=4) were slaughtered at different withdrawal periods and lung, kidney, liver and meat samples were taken, frozen and stored at -20°C until analysed.

RESULTS

Sample Pre-treatment

The sample pre-treatment for TMP and the sulfonamides was slightly different. The extraction was carried out with aqueous solutions at different pH to ensure that the analytes were in their dissociated form (in order) to facilitate extraction with an aqueous solvent. After extraction of muscle tissues with 0.2 M NaAc pH 8.6 the pH of the extract was usually approximately pH 6.0 and adjustment to pH 6.0 (in case of residues of SDM and AcSDM) was superfluous. Muscle, kidney and liver extracts prepared with 0.2 M NaAc pH 8.6 and adjusted to pH 4.6 or 6.0 with concentrated acetic acid tended to become turbid upon standing at room temperature. Turbidity could be avoided

if tissue extracts, after adjusment of pH, were partially deproteinized by warming at 37°C for 30 min. and subsequently centrifuged. After partial deproteinization, liver extracts were centrifuged at 5°C and temperature of the sample was kept at 5°C until analysed to avoid additional turbidity. Although no turbidity of the extracts occurred with tissues extracted with 0.2 M NaAc pH=5.0 at room temperature, samples were also kept at 5°C to ensure stability of the analytes.

Chromatography

The elution behaviour of drugs and metabolites on the GPC column was investigated. Therefore, the retention volumes of each drug and its corresponding metabolite(s) were determined. As an example, the influence of the molar concentration of the mobile phase applied for GPC on the retention volume and the resolution of a mixture of SMX and AcSMX are given in figure 2. An increase in the molar concentration of the mobile phase resulted in a decrease in the retention volume of the total eluate fraction and an increase in the resolution between SMX and AcSMX. The addition of 100 mM potassium chloride to 10 mM NaAc also resulted in a decrease of the retention volume and an increase in the resolution. The molar concentration chosen for further analysis was 50 mM NaAc. An increase in pH (4.6 → 6.0 → 7.0) resulted in a smaller retention volume for the sulfonamides and their metabolites, whereas TMP and its metabolites were more retained on the GPC column (not shown). A typical gel-permeation chromatogram of a standard solution and a spiked muscle extract at a concentration of 1 μg/g SDM and AcSDM is given in figure 3.

Drugs and metabolites could be quantitatively preconcentrated (loss < 5%) with the appropriate buffer solution (see table 1) applying flush volumes

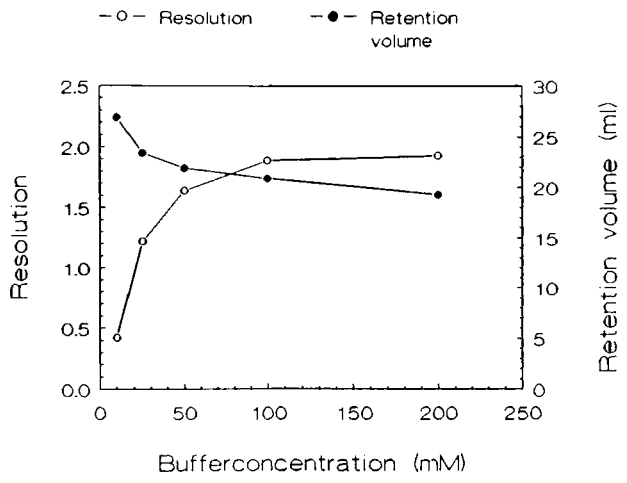

FIGURE 2. Relationships between the molar concentration of the mobile phase applied for GPC and the retention volume (●) or the resolution (o) of a mixture of SMX and AcSMX at a concentration of 1 µg/ml.

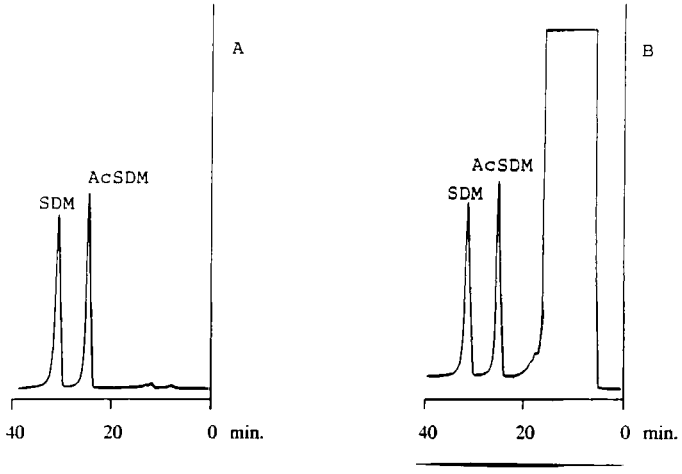

FIGURE 3. Chromatograms of a standard (A) and a spiked muscle extract (B) at a concentration of 1 µg/g SDM and AcSDM on the Progel G2000 SW gel-permeation column. The applied attenuation was 0.03 AUFS. For instrumentation and other chromatographic conditions see Materials and Methods.

TABLE 1

The applied eluents for GPC, preconcentration and reversed-phase HPLC

Analytes	GPC	Preconcentration	HPLC
SDM/AcSDM	0.05 M NaAc pH=6.0	0.05 M NaAc pH=6.0	0.05 M NaAc pH=6.0 / ACN* = 85 / 15
SMX/AcSMX	0.05 M NaAc pH=4.6	0.05 M NaAc pH=4.6	0.05 M NaAc pH=4.6 / ACN = 80 / 20
TMP/M1/M4	0.05 M NaAc pH=7.0	0.05 M NaAc pH=7.0	0.05 M NaAc + 0.2% TEA# pH=6.0 / ACN = 85 / 15

* ACN = Acetonitrile
\# TEA = Triethylamine

up to 30 ml. The packing material of the preconcentration column was renewed after approximately 25 injections. Deterioration of the preconcentration column became evident when compounds were not quantitatively retained anymore during preconcentration. Therefore, standard solutions were placed at the beginning and end of a series of samples. Reduction of the internal diameter of the preconcentration column to 2.1 mm negatively influenced the preconcentration of (especially) sulfonamides and their metabolites in kidney and liver samples. After trace enrichment was completed the analytes were completely desorbed from the preconcentration column with an eluent containing 10% or more acetonitrile after a backflush period of 2 min.

Typical HPLC chromatograms of blank muscle, kidney or liver tissue and tissues spiked at concentrations of 100 ng or 50 ng analyte/g tissue are given in figures 4, 5 and 6 respectively. Application of the Supelcosil LC-18-DB HPLC column resulted in better peak shapes of TMP and its metabolites

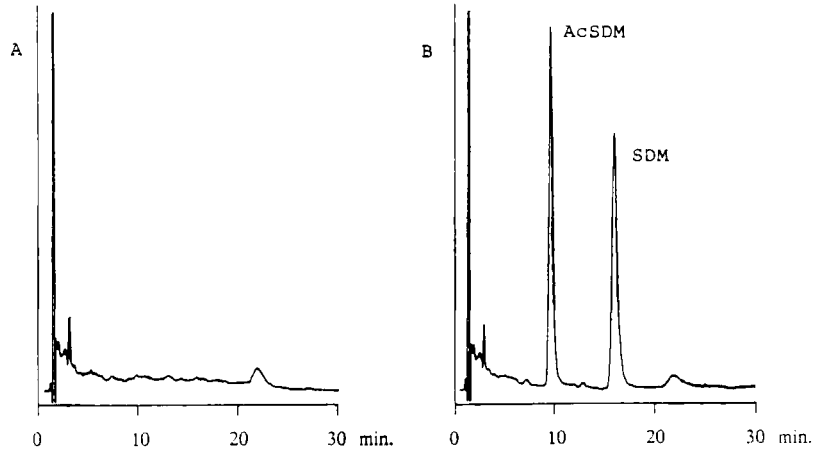

FIGURE 4. Chromatograms of a blank (A) and a spiked (B) muscle sample at a concentration of 100 ng SDM and AcSDM per g tissue. The analytical column was a ChromSpher C18 HPLC column. The applied attenuation was 0.004 AUFS. For instrumentation and other chromatographic conditions see Materials and Methods.

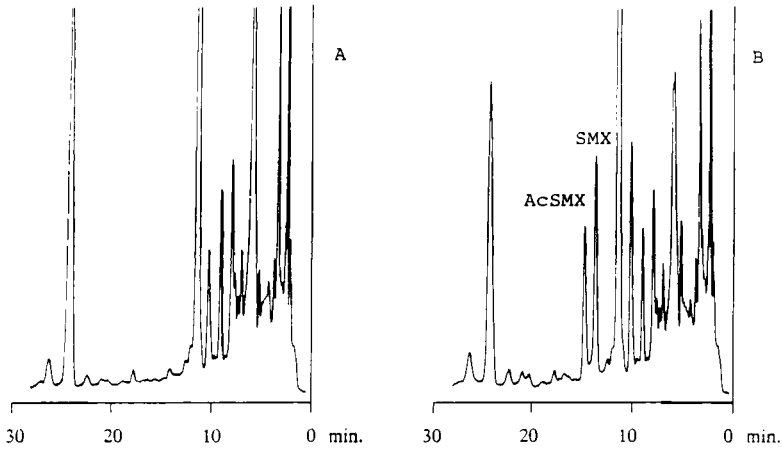

FIGURE 5. Chromatograms of a blank (A) and a spiked (B) kidney sample at a concentration of 50 ng SMX and AcSMX per g tissue. The analytical column was a Supelcosil LC-18-DB column. The applied attenuation was 0.004 AUFS. For instrumentation and other chromatographic conditions see Materials and Methods.

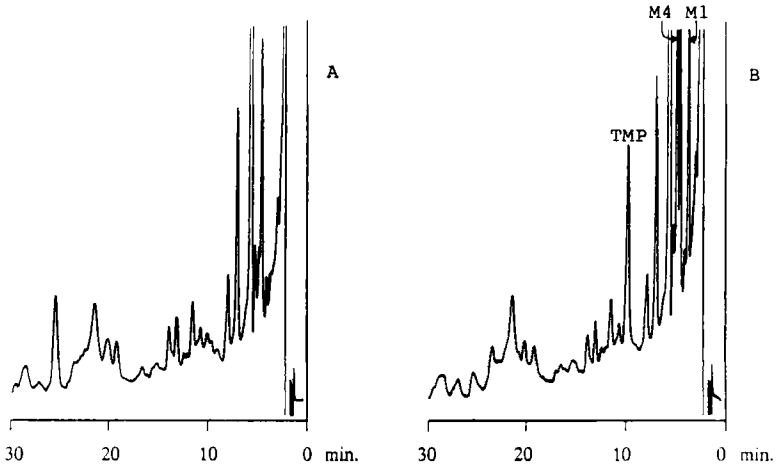

FIGURE 6. Chromatograms of a blank (A) and a spiked (B) liver sample at a concentration of 50 ng TMP, M1 and M4 per g tissue. The analytical column was a Supelcosil LC-18-DB column. The applied attenuation was 0.002 AUFS. For instrumentation and other chromatographic conditions see Materials and Methods.

(weak bases) but also in an improved resolution of the sulfonamides and their metabolites. The total analysis time of one sample ranges from 50 to 60 minutes. The analysis of a series of samples allows the introduction of a sample onto the GPC column at the moment the HPLC analysis of the preceding sample takes place, thereby reducing the analysis time to 35-45 minutes.

Recovery

The recoveries obtained for the investigated drugs and their metabolites in spiked muscle, kidney and liver tissue are given in tables 2 and 3. In the concentration ranges tested, the recoveries were found to be very constant for each tissue (coefficients of variation <8%). Recoveries for liver, kidney and

TABLE 2

Mean recoveries (n=6) and coefficients of variation (C.V.) of the sulfonamides and their *N*4-acetyl metabolites in spiked muscle, kidney and liver tissue. C.V. are given in parentheses.

Tissue	Concentration (μg/g)	SDM (%)	AcSDM (%)	SMX (%)	AcSMX (%)
Muscle	0.05	68.9 (3.4)	71.5 (3.1)	69.3 (4.2)	70.0 (3.1)
	0.1	74.2 (2.7)	72.3 (3.0)	63.3 (4.2)	64.9 (2.7)
	0.2	71.4 (1.9)	73.6 (2.2)	66.6 (1.4)	66.6 (1.3)
	1.0	72.4 (2.4)	70.1 (3.3)	67.9 (0.9)	62.8 (2.4)
Kidney	0.05	75.3 (4.3)	70.2 (2.2)	76.7 (1.7)	67.3 (1.5)
	0.1	77.2 (2.9)	74.1 (1.3)	81.7 (1.2)	69.9 (1.7)
	0.2	81.1 (1.1)	79.7 (0.9)	76.9 (2.3)	66.7 (2.6)
	1.0	84.9 (1.0)	81.5 (2.5)	74.8 (0.8)	65.0 (0.6)
Liver	0.05	67.5 (6.7)	65.5 (4.3)	65.5 (5.3)	60.3 (6.6)
	0.1	69.1 (4.3)	64.3 (2.3)	67.4 (6.7)	64.9 (4.5)
	0.2	72.3 (4.2)	69.0 (4.5)	65.7 (5.0)	69.2 (3.5)
	1.0	70.6 (3.3)	68.4 (3.9)	69.6 (4.8)	65.4 (3.7)

muscle tissue were carried out at concentrations 0.5, 1, 2, and 10 times the established tolerance level in The Netherlands. Recoveries for lung tissue were carried out in duplicate at different concentrations and were approximately the same as for muscle tissue.

Detection Limits

The limit of detection was defined as the amount of drug which resulted in a peak height three times that of the baseline noise in a blank tissue

TABLE 3

Mean recoveries (n=6) and coefficients of variation (C.V.) of trimethoprim and its demethyl metabolites in spiked muscle, kidney and liver tissue. C.V. are given in parentheses.

Tissue	Concentration (µg/g)	TMP (%)	M1 (%)	M4 (%)
Muscle	0.025	70.2 (4.1)	68.4 (4.9)	73.7 (4.3)
	0.05	73.3 (3.6)	73.0 (5.9)	72.3 (2.5)
	0.1	73.1 (3.7)	62.9 (4.3)	60.9 (4.4)
	0.5	73.4 (0.9)	67.9 (0.5)	69.3 (0.3)
Kidney	0.025	79.1 (3.5)	73.9 (3.9)	78.7 (3.0)
	0.05	67.7 (3.9)	71.1 (3.7)	69.0 (1.6)
	0.1	68.3 (4.6)	66.3 (4.4)	69.1 (3.0)
	0.5	79.6 (3.6)	79.6 (1.7)	75.8 (2.6)
Liver	0.025	76.6 (5.5)	77.0 (2.0)	69.0 (5.6)
	0.05	67.3 (3.7)	77.4 (1.5)	71.3 (2.5)
	0.1	81.5 (7.2)	75.4 (4.0)	69.4 (3.7)
	0.5	76.5 (1.0)	75.6 (0.5)	63.2 (2.2)

sample. The calculated limit of detection for parent drugs and metabolites in lung tissue was 50 ng/g. Detection limits (in ng/g) of sulfonamides and metabolites in meat, kidney and liver were respectively 10, 25 and 25 and for TMP and metabolites respectively 15, 25 and 25.

Specificity

Due to the different extraction and chromatographic separation steps for sulfonamides and TMP, no interferences between one analyte and another occurred.

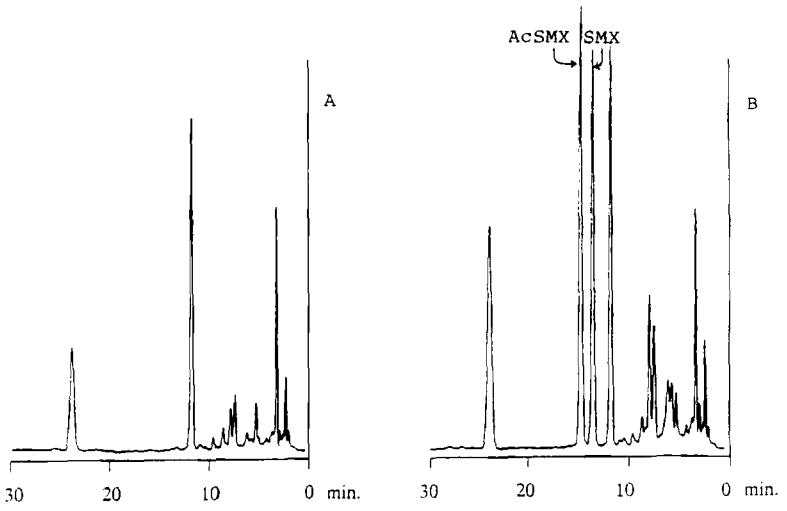

FIGURE 7. Characteristic chromatograms of kidney samples from treated pigs, slaughtered at 36 h (A) and 12 h (B) after the last feed medication. Sample pre-treatment and multi-dimensional LC was carried out for SMX and AcSMX. The analytical column was a Supelcosil LC-18-DB column. The applied attenuation was 0.02 AUFS. For instrumentation and other chromatographic conditions see Materials and Methods.

Interferences

Chromatograms of blank muscle, kidney or liver tissue are shown in figures 4A, 5A and 6A respectively. Because the investigated drugs and metabolites are ionizable compounds their chromatographic behaviour depends strongly on the pH of the eluent. Slight changes in the pH of the eluent have great impact on the retention times of the investigated compounds, regardless of the nature of the analytical reversed-phase column. For example, by increasing the pH of the mobile phase applied for the determination of SMX and AcSMX (table 1) from 4.6 to 5.6, AcSMX eluted

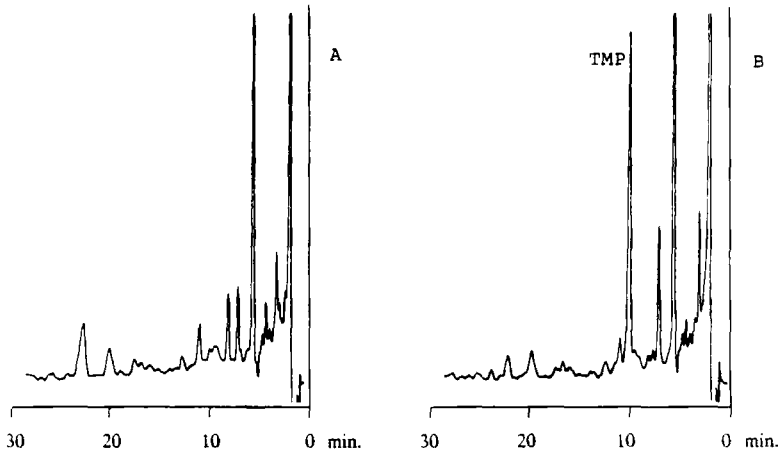

FIGURE 8. Typical chromatograms of kidney samples from treated pigs, slaughtered at 36 h (A) and 12 h (B) after the last feed medication. Sample pre-treatment and multi-dimensional LC was carried out for TMP, M1 and M4. The analytical column was a Supelcosil LC-18-DB column. The applied attenuation was 0.004 AUFS. For instrumentation and other chromatographic conditions see Materials and Methods.

before SMX [1]. Therefore, separation of interfering substances from the compound of interest could be achieved successfully with a minor adjustment of the pH.

Animal Experiments

Characteristic chromatograms of porcine kidney tissue collected during the animal experiment are shown in figures 7 and 8. They serve mainly as an illustrative example of the distinct presence of AcSMX and the absence of free (unconjugated) M1 and M4 in edible tissue after feed medication with 125 mg SMX in combination with 25 mg TMP per kg feed. The concentrations in

kidney for SMX, AcSMX and TMP in the presented chromatograms were respectively 0.42, 0.65 and 0.25 μg/g (Figures 7B and 8B). After a withdrawal period of 36 hours no drugs or metabolites could be detected (Figures 7A and 8A).

DISCUSSION

On-line combinations of GPC (or size-exclusion chromatography) and RPC for the determination of compounds in plasma or foods have been described [2-8]. In most of these studies the investigated compound(s) eluting from the gel-permeation (or size-exclusion) column were concentrated on top of the reversed-phase HPLC column (on-column concentration). Subsequent step or gradient elution of the second column separates the analyte(s) from compounds co-eluting from the first column. Sometimes only a small part of the selected eluate fraction of the gel-permeation column is introduced onto the reversed-phase column (heart-cutting technique), because the eluate fraction is too large for on-column concentration or the analyte(s) are not retained on the HPLC column by the mobile phase of the gel-permeation column. The disadvantage of the above-mentioned techniques is that the sensitivity is not high enough for our purposes. The combination of on-line gel-permeation chromatography and preconcentration provided clean chromatograms of blank edible tissues from pigs, resulting in low detection limits ($\leq$ 25 ng/g tissue). To our knowledge, no methods have yet been published on the liquid chromatographic determination of the above-mentioned drugs and metabolites in tissues with comparable low detection limits. Moreover, the described method was successfully applied to tissue samples collected during and after feed medication of healthy pigs (figures 7 and 8).

Applying GPC for the on-line removal of proteins of plasma samples prior to HPLC analysis of drugs has shown that drugs with different degrees of protein binding can be completely released from plasma proteins during their elution transport through the GPC column [8,9]. In these cases, the displacement of a drug from plasma proteins is achieved by the dilution of plasma in excess of buffer, the change in pH, the addition of salts or by any combination of these possibilities. The presented method resulted in reproducible recoveries which were independent of the different plasma protein binding values of the investigated drugs. This indicates that drugs were displaced from tissue proteins during extraction and/or during elution on the GPC column.

The fact that the investigated drugs and their metabolites did not have the same elution volume, as was expected on basis of their low molecular weight, indicates mixed retention mechanisms (GPC and adsorption). The elution volumes of the total eluate fraction and the resolution between a drug and its metabolite were influenced by the pH and the molar concentration of the eluent. Although the occurence of adsorption gives rise to a better resolution between analytes and proteins it excludes the collection of all low molecular weight compounds (i.e. most drugs) in a small defined fraction. Nevertheless, the applicability of on-line preconcentration provides the required trace enrichment of large eluate fractions. Because drugs and metabolites were slightly retained on an additional gel-permeation guard column (retention volume ± 4 ml) the proposed set-up, as shown in figure 1B, can also be applied for the determination of drugs and their metabolites in crude aqueous tissue extracts. Whether this ensures a prolonged life-time of the GPC column is presently being investigated at our laboratory.

In order to avoid precipitation of tissue proteins on top of the GPC column or irreversible adsorption on the column packing material, the pH of the applied extract was adjusted to the pH of the mobile phase applied for GPC. For the same reason the pH of the buffer solution used for

preconcentration was similar to the pH of this aqueous mobile phase. It was found that preconcentration does not necessarily has to be carried out at a pH lower than the pK_a of the investigated (weak) acids (sulfonamides and their metabolites) or higher than the pK_a of the investigated (weak) bases (TMP and its metabolites). Apparently the analytes need not to be in their undissociated form in order to be quantitatively adsorbed on the polymeric, apolar material used for preconcentration.

In conclusion, the technique of multi-dimensional LC, combining aqueous gel-permeation on-line with trace enrichment and reversed-phase chromatography, is a feasible approach for the analysis of residues of veterinary drugs in tissues. Moderately polar compounds, (weak) acids and (weak) bases can be determined on the presented LC system. The determination of non-ionizable, lipophilic compounds requires different mobile and stationary phases.

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