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DETERMINATION OF RESIDUES OF CARBADOX AND SOME OF ITS METABOLITES IN SWINE TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ON-LINE PRE-COLUMN ENRICHMENT AND POST-COLUMN DERIVATIZATION WITH UV–VIS DETECTION

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SUMMARY

A high-performance liquid chromatographic (HPLC) method that uses UV–VIS detection and post-column derivatization with sodium hydroxide was developed for the determination of the growth-promoting antibiotic carbadox and three of its metabolites in swine muscle, liver and kidney tissues. Sample pre-treatment involves extraction with methanol–acetonitrile, purification over an alumina–Florisil column and partition with isooctane. A 2-ml volume of the final aqueous extract is injected into a column-switching HPLC system; detection is performed at 420 nm. The limits of determination are in the range 1–5 μg/kg. Preliminary experiments show a good precision with mean recoveries of 81–87% and a coefficient of variation of 4–10%. The method is highly selective and can be used in routine monitoring programmes.

INTRODUCTION

Carbadox (CBX) methyl 3-(2-quinoxalinylmethylene)carbazate-N^1, N^4-dioxide, is used extensively in veterinary practice as a growth-promoting and chemotherapeutic agent for young swine. The structures of the parent drug and some of its metabolites are given in Fig. 1. Carbadox is administered through the feed at levels of 50–150 mg/kg. To ensure the absence of residues of CBX and its metabolites from edible products, a withdrawal period of 4 weeks has been established. Further, CBX should not be administered to swine over 4 months old.[1].

Metabolism studies have shown that CBX is rapidly converted into monooxy and desoxy metabolites. Quinoxaline-2-carboxylic acid (QCA) is considered to be the last remaining major residue and may serve as a marker substance[2]. However, CBX and desoxy-CBX are considered to be carcinogenic and mutagenic[2,3] and methods should be available to monitor even very low concentrations in edible tissues.

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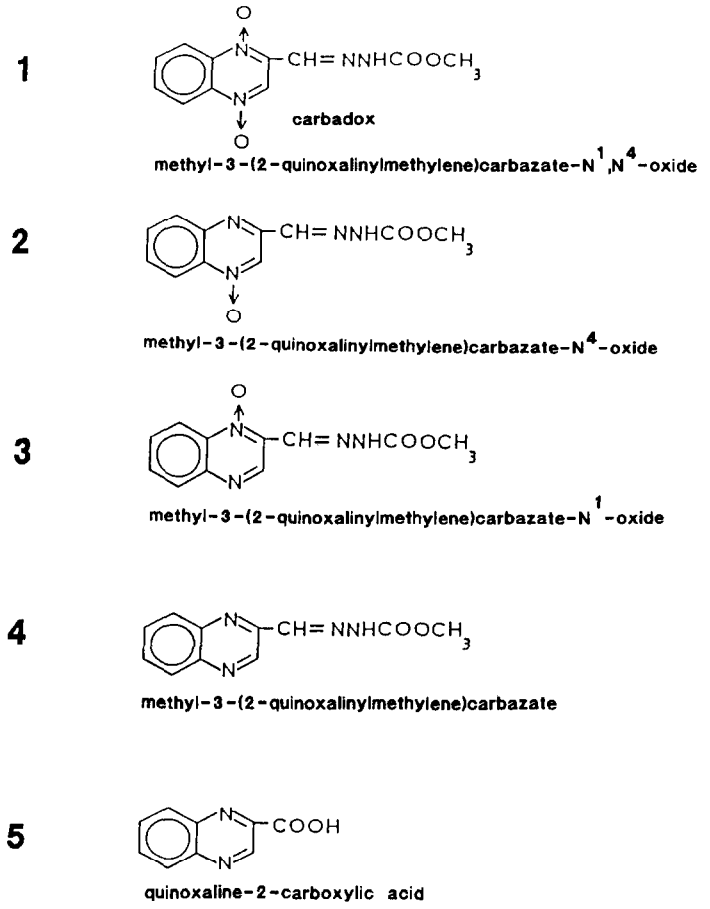

Fig. 1. Molecular structures of carbadox (1) and its monoxy (2 and 3), desoxy (4) and quinoxaline acid (5) metabolites.

High-performance liquid chromatographic (HPLC)[4–9], polarographic[10–12], thin-layer chromatographic (TLC)[13] and gas chromatographic (GC)[14] methods have been described for the determination of residues of CBX and/or its metabolites in various matrices, including feeds[8,9]. CBX has been detected electrochemically[10–12,15] and using fluorescence[4,5,16] or UV–VIS absorption spectrometry[5–9,17–21]. In some studies no chromatographic separation was performed before the actual determination[10–12,15,19–21]. A few workers have also studied the analysis of some metabolites. The supposed final metabolite QCA can be detected electrochemically[10–12] or, after derivatization, with electron-capture detection or mass spectrometry in the selected ion mode[14]. The monoxy- and desoxy-CBX metabolites can be detected by UV–VIS spectrometry at 280 or 350 nm[5,6], but our preliminary experiments showed no active fluorescence.

All of the relevant residue methods mentioned require extensive sample clean-up to remove interfering components and/or to hydrolyse CBX-related residues to QCA[6,11–14]. Often a number of liquid–liquid extraction and off-line column puri-

fication steps are performed, resulting in the analysis of only a few samples per day. The limits of detection lie in the range 5–40 μg/kg for CBX and desoxy-CBX and 30–100 μg/kg for QCA.

Previous methods do not provide the desired limits of detection and are not always practical or suitable for metabolite analysis. Therefore, in this work a routine method was developed for the determination of CBX and a number of relevant metabolites in swine tissues at levels of 1–5 μg/kg.

EXPERIMENTAL

Chemicals and reagents

All chemicals were of analytical-reagent grade (Merck, Darmstadt, F.R.G.). Standard carbadox was obtained from Pfizer (Groton, CT, U.S.A.) and standard solutions of methyl 3-(2-quinoxalinylmethylene)carbazate (desoxy-CBX), methyl 3-(2-quinoxalinylmethylene)carbazate N^1-oxide (N^1-monooxy-CBX) and methyl 3-(2-quinoxalinylmethylene)carbazate N^4-oxide (N^4-monooxy-CBX) were a gift from the Central Veterinary Institute (Lelystad, The Netherlands).

Florisil (0.075–0.150 mm) was purchased from Merck and alumina (Woelm neutral, activity 1) (Art. No. 02090) from Woelm Pharma (Eschwege, F.R.G.). The extraction solution was acetonitrile–methanol (1:1). The HPLC eluent was prepared by mixing 850 ml of 0.01 *M* sodium acetate buffer (adjusted to pH 6 with acetic acid) with 150 ml of acetonitrile. Water was purified with a Milli-Q purification system (Millipore, Milford, MA, U.S.A.). Stock standard solutions contained 100 μg/ml in acetonitrile–methanol (1:1) and working standard solutions had concentrations of 0.001, 0.005 or 0.01 μg/ml in water.

Instrumentation and chromatographic conditions

The column-switching HPLC system depicted in Fig. 2 consisted of two liquid chromatographic pumps capable of maintaining a constant pulseless flow-rate of

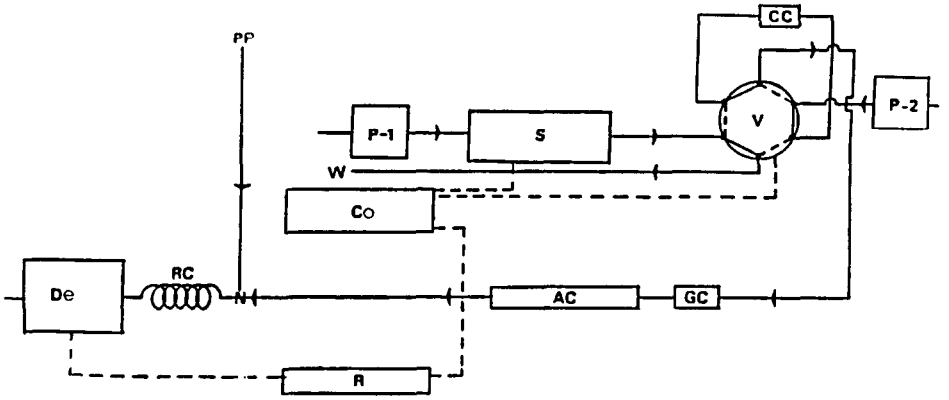

Fig. 2. Schematic representation of the HPLC system used. The sample is introduced with pump P-1 and sampler S to the column-switching sample enrichment module with the six-port valve V and enrichment column CC. After the HPLC separation using pump P-2, guard column GC and analytical column AC, the sample passes the post-column derivatization unit with the peristaltic pump PP and the reaction coil RC and is detected by the UV–VIS detector De. Peaks are recorded by recorder R. All timed events are handled by a microprocessor Co.

0.2–2 ml/min [Model 6000 A (Millipore) and Spectroflow 400 (ABI-Kratos, Ramsey, NY, U.S.A.)]. A Model 7125 fixed-loop (2 ml) injector (Rheodyne, Berkeley, CA, U.S.A.), an automatic six-port valve (Rheodyne 7010), a Model 450 controller (Kratos), a UV–VIS spectroflow 783 absorbance detector (Kratos), an M 2002 peristaltic tubing pump (Skalar, Breda, The Netherlands), a 1/16 in. × 0.75 mm tee (Valco, Houston, FL, U.S.A.) and a 2 m × 0.5 mm I.D. knitted PTFE reaction coil were used. The analytical column was a 200 × 3 mm I.D. cartridge containing 5-μm ChromSpher C_{18} (Chrompack, Middelburg, The Netherlands) and the guard column was a 10 × 2.1 mm I.D. cartridge containing 37–50-μm Bondapak C_{18}/Corasil (Millipore). The enrichment column used in the final procedure was a 60 × 4.6 mm I.D. stainless-steel column containing 37–50-μm Bondapak C_{18}/Corasil.

During method development, 60 × 4.6 mm I.D. or 10 × 2.1 mm I.D. columns containing 53-μm Partisil ODS-3 (Whatman, Clifton, CA, U.S.A.) or the polymeric materials 50–100-μm XAD-4 (Serva, Heidelberg, F.R.G.) or 15–25-μm PRP-1 (Polymer Labs., Amherst, MA, U.S.A.) were tested. Also, a 150 × 4.6 mm I.D. analytical column containing 5-μm Supelcosil LC-8 DB (Supelco, Bellefonte, PA, U.S.A.) was tested. A Model 400 stomacher laboratory blender (Lameris, The Netherlands), a Pierce (Rockford, IL, U.S.A.) evaporator and an MSE-coolspin centrifuge (MSE Crawley, Sussex, U.K.) were used. The eluent flow-rate was 0.6 ml/min, the sample enrichment flow-rate was 0.5 ml/min and the derivatization reagent flow-rate was 0.23 ml/min. The timed events sequence used for enrichment and separation was as follows: 0 min, start sampling and flushing; 20 min, activate six-port valve for back-flushing and set detector at auto-zero; 25 min, reset six-port valve; 35 min, new sample.

Sample preparation

Caution: solutions of CBX and its metabolites are light-sensitive and therefore all handling should be performed under artificial yellow light and amber glassware should be used.

Accurately weigh *ca.* 10 g of homogenized sample (muscle, liver, kidney) in a 1000-ml stomacher bag. Add 40.0 ml of acetonitrile–methanol (1:1) and blend for 3 min. Transfer the crude extract into a centrifuge tube and centrifuge for 5 min at 2000 *g*. Apply the supernatant to an alumina–Florisil column prepared by pouring first 8 g of alumina and then 2 g of Florisil into a 400 × 10 mm I.D. glass column, and collect the first 10 ml of eluate in a calibrated tube. Evaporate the solvent with a gentle stream of nitrogen at 40–50°C to a volume of 1–1.5 ml. Dilute to 4.0 ml with water and mix. Extract this solution with 2 ml of isooctane, centrifuge for 5 min at 2000 *g* and inject 2.0 ml of the aqueous phase on to the HPLC column.

RESULTS AND DISCUSSION

General

In multi-component residue analysis in complex matrices, the selective detection of the analytes is one of the major problems. This also applies to the determination of carbadox and its metabolites in swine tissues. Therefore, after the HPLC separation conditions have been established, this aspect will be discussed before the sections dealing with extraction, purification and sample enrichment. As selective

UV–VIS detection of the more persistent, but toxicologically not very relevant, metabolite QCA proved to be impossible, as is shown in the post-column derivatization section, this metabolite was not evaluated further in the extraction, purification and enrichment experiments.

Chromatography

In the literature, most eluent systems used for the reversed-phase separation of CBX and related compounds are composed of a mixture of acetonitrile and water or an acidic (pH 3–4) buffer[4,5,8,16,18]. De Graaf *et al.*[5] used a linear acetonitrile–water gradient to separate CBX and desoxy-CBX from gastrointestinal tract matrix interferences. MacIntosh and Neville[6] were able to separate CBX, desoxy-CBX and N^1- and N^4-monooxy-CBX with an acetonitrile–ammonium acetate buffer–ethanol (pH 6.8) eluent on a C_8-bonded phase. With the system used in our laboratory for the determination of CBX in feeds, the complete resolution of CBX, N^1- and N^4-monooxy-CBX and desoxy-CBX was achieved on a C_{18}-bonded phase with an even simpler eluent, acetonitrile–0.01 *M* sodium acetate buffer (pH 6) (15:85) (Fig. 3). This system was used in this present study.

Two types of analytical columns were tested: a 150 × 4.6 mm I.D. column containing Supelcosil LC-8 DB and a 200 × 3 mm I.D. cartridge column containing Chromsep C_{18}. The columns showed similar resolution but, probably owing to the smaller inner diameter of the cartridge column, the peak broadening was less in the

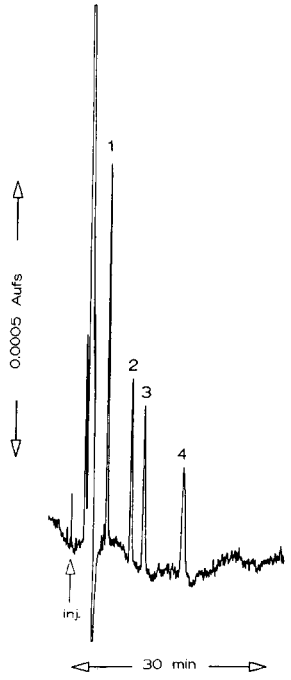

Fig. 3. HPLC trace obtained after injection of 50 µl of a standard solution containing 2.5 ng of each carbadox and its monooxy and desoxy metabolites. For experimental conditions, see text. Peaks: 1 = carbadox; 2 = N^4-monooxy; 3 = N^1-monooxy; 4 = desoxy.

latter instance, resulting in a 50% increase in the peak response. This phenomenon, originating from technical features rather than characteristics of the packing material, was observed earlier in another residue method development study[22].

Post-column derivatization

Carbadox and its metabolites show UV–VIS absorption maxima at wavelengths ranging from 275 to 380 nm. In Fig. 4 the UV–VIS spectra of CBX, desoxy-CBX and QCA are shown. For N^1- and N^4-monooxy-CBX, an insufficient amount of material was available to measure their UV–VIS spectra. As shown by De Graaf *et al.*[5] and MacIntosh and co-workers[6,7], extensive sample clean-up is necessary to remove UV-absorbing interferences when CBX or its metabolites are determined in biological matrices and detected at 280–380 nm. Therefore, a more selective detection method was necessary.

In feed analysis, a number of direct UV–VIS spectrometric methods have been developed for the determination of high levels of CBX (50–150 mg/kg). The final step in these procedures is the reaction of CBX with 0.1 *M* sodium hydroxide[19–21]. The yellow reaction product is detected at 420 nm, thereby strongly reducing feed matrix interferences. The compound formed is not very stable and has not been characterized. The sodium hydroxide reaction is, however, fast and reproducible. This opened up the possibility of a post-column reaction with which a well defined, short reaction time and a reproducible time interval between reaction and detection can be achieved. In Fig. 4, the UV–VIS spectra of CBX and its metabolites after the addition of 0.5 *M* sodium hydroxide are also shown. Both CBX and desoxy-CBX show a shift of their absorption maxima to higher wavelengths; QCA apparently does not give a similar

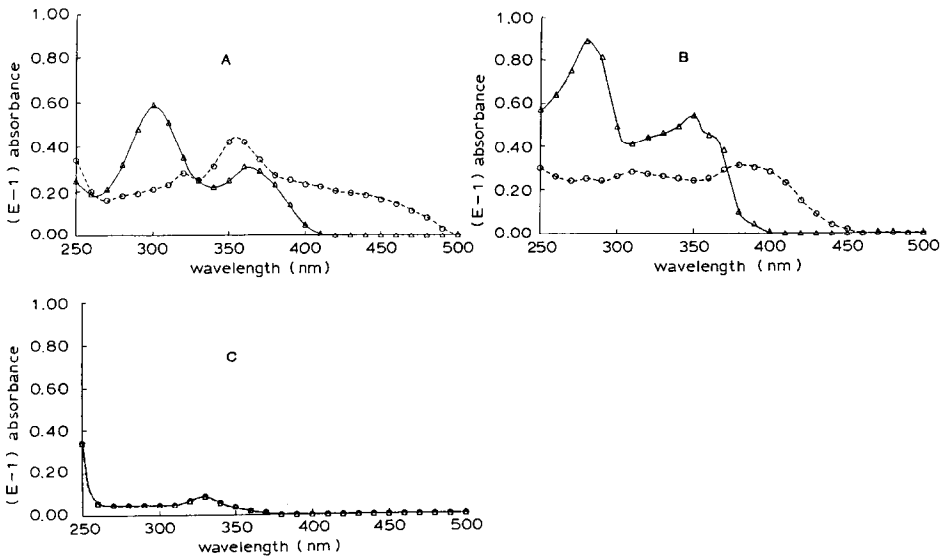

Fig. 4. UV–VIS spectra of (A) carbadox, (B) desoxy-carbadox and (C) quinoxaline-2-carboxylic acid dissolved in the mobile phase both before (△) and after (○) the addition of 0.5 *M* sodium hydroxide. Conditions: path length, 1 cm; analyte concentration, 100 μg/ml.

reaction with sodium hydroxide. This indicates that the carbazate moiety is essential for the reaction, or presumably both monooxy-CBX metabolites will also react.

As the UV maximum of QCA is near 245 nm and the compound is very polar, it will be extremely difficult to detect QCA selectively in the presence of tissue matrix components using HPLC with UV–VIS absorption detection. Therefore, QCA was not evaluated further in this study. The choice of 420 nm as the detection wavelength was a compromise between maximal adsorbance of the analytes and minimal interferences by matrix components. Addition of sodium hydroxide increases the molar absorptivity at 420 nm from less than 10^2 to $1.5 \cdot 10^4$ for CBX and from less than 10^2 to $2.2 \cdot 10^4$ for desoxy-CBX. Usually, CBX is detected at its second UV maximum at 365 nm and not at its higher maximum at 280 nm, because there are fewer interferences at 365 nm.

As can be seen from Fig. 4, the absorptivity at 420 nm after sodium hydroxide addition is essentially the same as that obtained at 365 nm without sodium hydroxide addition. In other words, the intrinsic sensitivity remains the same, but the selectivity is improved.

The sodium hydroxide reaction was tested in the post-column derivatization system by mixing the reagent with the column effluent and letting them react in a knitted PTFE coil at room temperature. The sodium hydroxide concentration was varied from 0.1 to 1.0 *M* to optimize the response and noise. A 0.5 *M* reagent concentration proved to be optimal (Fig. 5A), giving a compromise between noise and response. To test the time dependence of the reaction, coils of different lengths, *i.e.*, with different residence times, were tested (Fig. 5B). The optimal response was obtained with a 2 m × 0.5 mm I.D. coil or a 28-s residence time.

Extraction and sample clean-up

In most of the extraction and purification experiments, CBX was used as a model compound. Because CBX is more polar than its metabolites, it can be considered to be a good indicator substance for the monooxy and desoxy-metabolites.

Preliminary experiments using a very simple aqueous extraction of animal tissue, followed by on-line dialysis and HPLC with pre-column enrichment and post-column sodium hydroxide derivatization, in analogy with a method developed for sulphonamides in biological samples[23], showed that, in principle, the fully automated determination of CBX was possible but that the limit of detection was high (about 25 μg/kg). This was probably due to the limited efficiency of the on-line dialysis and not to poor extraction from the sample. Therefore, another approach was followed, using an organic sample extraction. Carbadox is readily soluble in various organic solvents, and reported extractants include dimethylformamide[5,20], ethanol[6], chloroform–methanol (3:1)[19], dichloromethane[4], acetonitrile–methanol (1:1)[17,24] and 2% ammonia in acetone[8]. In our department acetonitrile–methanol (1:1) had been used for several years in feed analysis, where it has shown to give 100% recovery[24]. Further, acetonitrile and methanol are efficient deproteination reagents that yield well separated phases after extraction. Therefore, acetonitrile–methanol (1:1) was used further in this study.

With a practical sample size of 10 g, 40 ml of acetonitrile–methanol (1:1) gave over 95% recovery for spiked (1–100 μg/kg tissue) CBX and desoxy-CBX samples. The extraction was performed with a stomacher blending apparatus. Stomacher

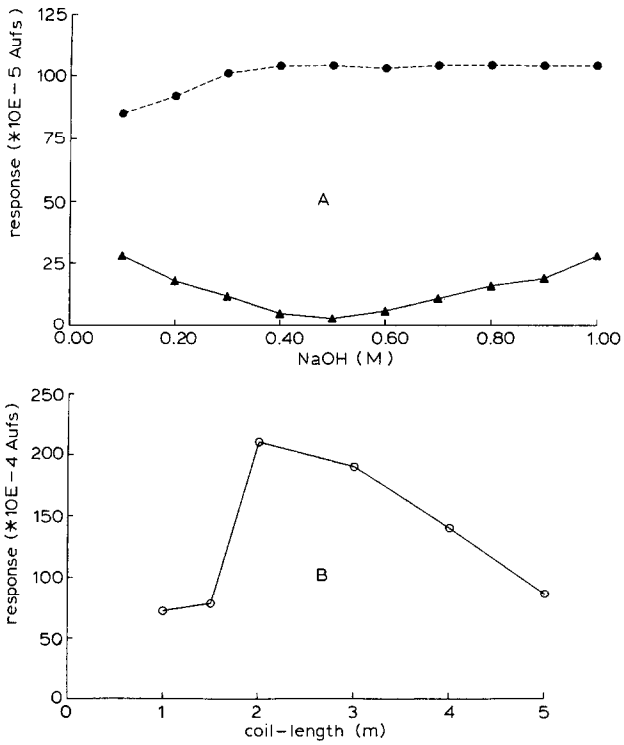

Fig. 5. (A) Plot of the response (●) and system noise (▲) obtained at 420 nm after injection of 20 ng of CBX into the column-switching HPLC system *versus* the concentration of the sodium hydroxide solution added post-column to the analytical column effluent. Reagent flow-rate, 0.23 ml/min. (B) Plot of the response obtained at 420 nm after injection of a carbadox standard solution into the HPLC system *versus* the length of the 0.5 mm I.D. knitted PTFE coil used in the post-column derivatization with 0.5 *M* sodium hydroxide.

blending if often used in microbiological analysis. The sample is introduced in a polypropylene bag and blended with the solvent without direct physical contact with instrument parts. Also, the bags are used only once. Hence, compared with blending with, for instance, an Ultra-Turrax, the risk of cross-contamination is strongly reduced.

After centrifugation of the crude extract, the supernatant (about 30 ml) was purified over an alumina–Florisil column to remove fat and colouring agents. CBX and its metabolites were not adsorbed on the column because of the water present in the extract, originating from the tissue sample. Different amounts of alumina (0–8 g) were tested with, always, 2 g of Florisil. Although the extracts became cleaner when the amount of alumina was increased, an amount of 8 g was chosen as a practical upper limit because otherwise too much extraction liquid was sorbed on to the column material. When 8 g of alumina were used, a maximum of 20 ml of eluate could be collected. Of these 20 ml, the last 10 ml eluted much more slowly than the first 10 ml. Fractionation of the eluate showed that the CBX concentration was slightly (10%) higher in the first 2 ml, and also that this first fraction produced cleaner chromatograms (see below). However, in order to achieve the intended low detection

levels, it was necessary to collect a minimum of 10 ml of eluate. This corresponded to about 2.5 g of tissue sample or to 2.5 ng of CBX when the tissue sample contained 1 μg/kg of CBX. Collection of more eluate increased the amount of CBX, but on the other hand resulted in a more time-consuming purification and subsequent evaporation of the eluate. When the eluate fraction was evaporated to dryness, dissolution of CBX became irreproducible. Therefore, the 10 ml of eluate were gently evaporated until essentially all organic solvent had been removed and about 1.5 ml of aqueous phase remained.

After evaporation, the aqueous residue was diluted to 4 ml with water. This allowed duplicate injection in the HPLC analysis and improved the reproducibility of the procedure.

When 2 ml of the final 4 ml of solution were injected into the final column-switching HPLC system, irreproducibly eluting components, interfering with CBX or its metabolites, were sometimes observed. In a later stage of this study, these interferences could be effectively removed by a simple extraction with 2 ml of isooctane. Isooctane is almost immiscible with water and CBX and its metabolites do not dissolve in it. Fig. 6 shows the effect of the isooctane extraction on the chromatograms obtained from different eluate fractions of blank liver samples. It allows the use of a five-fold increased sample amount without an increase in the matrix interferences.

The use of an internal standard was not considered practical, because it could interfere with the detection of unknown metabolites or other veterinary drugs (see also under *Characteristics and applications of the analytical method*).

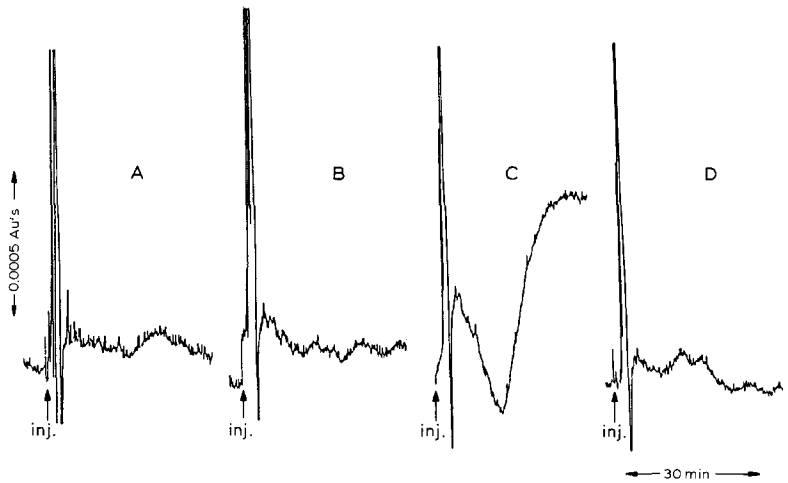

Fig. 6. Chromatograms obtained with blank swine liver samples using the final extraction and purification procedure, except for variations in the alumina–Florisil eluate fraction and isooctane extraction. (A) First 2 ml of eluate; without isooctane extraction. (B) First 2 ml of eluate; with isooctane extraction. (C) First 10 ml of eluate; without isooctane extraction. (D) First 10 ml of eluate; with isooctane extraction. In each instance the eluate was evaporated and diluted to 4 ml with water before injecting 2 ml into the HPLC system.

On-line sample enrichment and purification

Normally, a sample volume of only up to about 200 μl can be injected directly on to a conventional 4.6 mm I.D. HPLC column without too much additional peak broadening. Therefore in residue analysis, in general, trace enrichment is necessary during sample pre-treatment. In our study, an off-line sample enrichment factor of only about 2.5 was achieved on going from the crude extract mixture to the final aqueous HPLC injection solution, as discussed above. Additional off-line enrichment would have required time-consuming and recovery-lowering procedures. Therefore, an alternative approach was chosen, using on-line HPLC purification and enrichment with short pre-columns, similar to a recently described study[23].

Two types of stainless-steel enrichment columns were tested: 10 × 2.1 mm I.D. and 60 × 4.6 mm I.D. The columns were packed with chemically bonded silica material such as 53-μm Partisil ODS-3 and 37–50-μm Bondapak C_{18}/Corasil and with polystyrene–divinylbenzene copolymers such as 50–100-μm XAD-4 and 15–25-μm PRP-1. Material with relatively large particle sizes was chosen to prevent column clogging due to residual matrix components present in the final extract, and to permit the use of a low-pressure pump to introduce the samples because a second HPLC delivery pump will not always be available.

The enrichment characteristics of the various materials were tested with CBX and desoxy-CBX as model compounds, these being the most polar and apolar compound, respectively.

Carbadox enrichment. Aliquots of 2 ml of a standard solution containing 20 ng were injected on to the enrichment column. After injection, the column was flushed with a variable volume (2–16 ml) of water. Next, the column was backflushed to the analytical column with eluent for 5 min, and the CBX response (as peak height) was determined after post-column derivatization. On each column tested, a series of 2-ml injections was applied using increasing flushing volumes. A decrease in the response

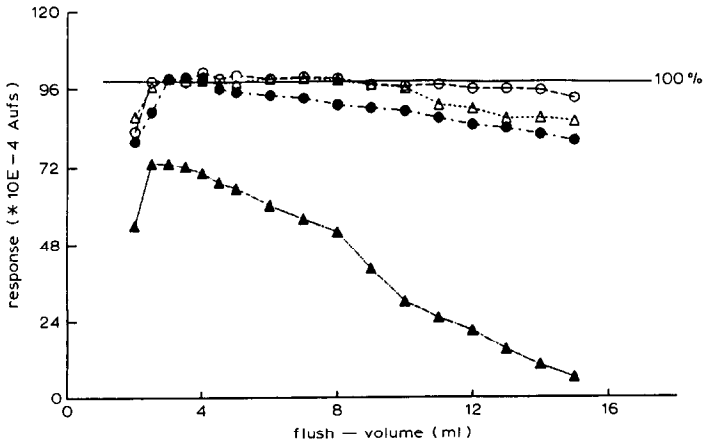

Fig. 7. Plot of the response at 420 nm obtained when 2-ml injections of a standard solution of carbadox (containing 20 ng) were applied to 60 × 4.6 mm I.D. enrichment columns filled with various packing materials *versus* the aqueous flushing volume applied before CBX was back-flushed to the analytical column. The straight line represents the response obtained after a direct 50-μl injection of 20 ng of CBX. For experimental conditions, see text. (○) Partisil ODS-3; (△) μBondapak C_{18}; (●) PRP-1; (▲) XAD-4.

at higher flushing volumes indicated that CBX was no longer fully retained on the column and/or that peak broadening occurred. Fig. 7 shows examples of response curves obtained according to this procedure for CBX on 60 × 4.6 mm I.D. columns. For comparison, the response obtained when 20 ng of CBX were introduced directly on to the analytical column as a 50-μl injection, without pre-column enrichment, is also shown. As can be seen, only the coarse polymerix XAD-4 material failed to retain CBX fully, and it also gave some additional peak broadening as indicated by the peak width at half-height, which increased by 15% on going from a flushing volume of 4 ml to 15 ml. The C_{18}-bonded phases all show good retention. This may be partly due to hydrogen bond interaction with the silanol groups (see below).

Desoxy-CBX enrichment. The retention behaviour of desoxy-CBX was tested in a similar way, but with fewer data points. In Table I the responses obtained after pre-column enrichment with three different aqeous flushing volumes are presented as a percentage of the maximum response obtained with a 50-μl direct injection. The desoxy-CBX results are comparable to those for CBX with regard to the behaviour on the C_{18} materials. However, with both polymer materials low responses are found, which indicates that polar interactions play an important role in the retention process.

TABLE I

EVALUATION OF ENRICHMENT PROCEDURE FOR DESOXY-CBX

Conditions: 20 ng of desoxy-CBX injected as a 2-ml injection on various 60 × 4.6 mm I.D. enrichment columns. Response compared with direct injection of 20 ng in 50 μl on to the analytical column.

Aqueous flushing volume (ml)	*Relative response (%) for column packed with*			
	XAD-4	*PRP-1*	*Bondapak-C_{18}*	Partisil ODS-3
2.5	50	50	100	100
10.0	55	65	100	100
15.0	55	65	100	100

The results imply that the N^1- and N^4-monooxy-CBX metabolites, with intermediate polarity, will probaly also be fully retained on the C_{18} bonded phases.

The Bondapak/Corasil C_{18} material was selected for further study because it is spherical and therefore generates less back-pressure than does Partisil, which is irregularly shaped.

Enrichment-column dimensions. Essentially, the retention characteristics of the 10- and 60-mm cartridges were similar under the experimental conditions used when standard solutions were injected. However, when spiked tissue extracts were injected, the 10-mm columns retained only about 30% of the CBX. Probably, surface modification of the small column due to sorption of residual matrix components partly blocks adsorption sites. The 60-mm columns, which contain 30 times more packing material, did not present this problem, as indicated by the 97–100% response obtained when CBX, N^4- and N^1-monooxy-CBX and desoxy-CBX were added to kid-

ney extracts (in the final 4 ml of aqueous phase) and analysed according to the pre-column enrichment HPLC procedure. A summary of the final analytical procedure is presented in Table II.

TABLE II

SUMMARY OF THE FINAL ANALYTICAL PROCEDURE

Parameter/step	*Value/conditions*
Sample size	10 g
Extraction	40 ml of acetonitrile–methanol (1:1)
Alumina–Florisil clean-up	Collect first 10 ml of eluate
Evaporate and dilute to	4 ml with water
Partition with	2 ml of isooctane
Inject	2 ml into the HPLC system (Fig. 2)
Enrichment-flush water flow-rate	0.5 ml/min
Enrichment column	60 × 4.6 mm I.D. 37–50-μm C_{18}-Bondapak/Corasil
Flushing time	20 min
Back-flushing time	5 min
Eluent	Acetonitrile–0.01 *M* sodium acetate, pH 6 (15:85)
Eluent flow-rate	0.6 ml/min
Analytical column	200 × 3 mm I.D., 5-μm Chromspher-C_{18}
Derivatization reagent	0.5 *M* sodium hydroxide
Derivatization flow-rate	0.23 ml/min
Reaction coil	2 m × 0.5 mm I.D. knitted PTFE
Detection	420 nm, 0.001 a.u.f.s.

Characteristics and applications of the analytical method

Preliminary precision and selectivity experiments were performed using the final method. Swine muscle, kidney and liver samples were spiked by injection of an aliquot (<0.5 ml) of a standard solution to the homogenized tissue sample and equilibration for 15 min. The samples were fortified with 1, 5 and 10 μg/kg of each CBX and desoxy-CBX. The amount of monooxy metabolites available during the study was not sufficient to include them in these spiking experiments. The results of the recovery experiments were pooled because no significant differences between matrices were observed. For CBX a mean recovery of 81% (coefficient of variation, C.V. = 10.3%; n = 12) was found for the 1, 5 and 10 μg/kg experiments. For desoxy-CBX a mean recovery of 87% (C.V. = 4.1%; n = 8) was found for the 5 and 10 μg/kg levels. A small positive bias, resulting from the slightly higher CBX concentration in the first 2 ml of alumina–Florisil eluate, is included in the observed recoveries. The high recoveries that were obtained indicate that no substantial analyte losses occur during the spiking and sample clean-up procedures (*i.e.*, enzymatic conversion in liver). However, future experiments will include stability studies for biological matrices. For CBX, 1 μg/kg could be determined in all matrices; for desoxy-CBX, however, 1 μg/kg was below the limit of detection. As the number of experiments was limited, an accurate calculation of the limit of detection was not possible. On the basis of the chromatograms obtained and with the equipment used, the estimated limits of detec-

tion are 0.5–1 μg/kg for CBX and 2–3 μg/kg for desoxy-CBX in liver, muscle and kidney. Preliminary experiments indicate that the limits of detection of the N^1- and N^4-monooxy-CBX metabolites are 1–2 μg/kg.

Fig. 8 shows typical chromatograms for blank and spiked muscle, liver and kidney samples. Very clean chromatograms were obtained even at the very sensitive detector setting of 0.001 a.u.f.s. Obviously, the combination of detection at 420 nm and off-line plus on-line sample purification removes interferences due to lipids, proteins, colouring agents or other animal tissue components very effectively. The method also seems applicable to other biological matrices.

About 20 samples can be analysed per day, which makes the method suitable for monitoring, surveillance and pharmacokinetic studies. In order to test the selectivity of the method, a number of veterinary drugs were injected into the HPLC system at concentrations corresponding to about 100 μg/kg of tissue. None of the compounds listed in Table III interfered in the determination of CBX or its N^1- and N^4-monooxy of desoxy metabolites. The nitrofuran drugs furazolidone, nitrofurazone, furaltadone and nitrofurantoine, however, also reacted with sodium hydroxide and eluted between CBX and desoxy-CBX, thus potentially interfering with the determi-

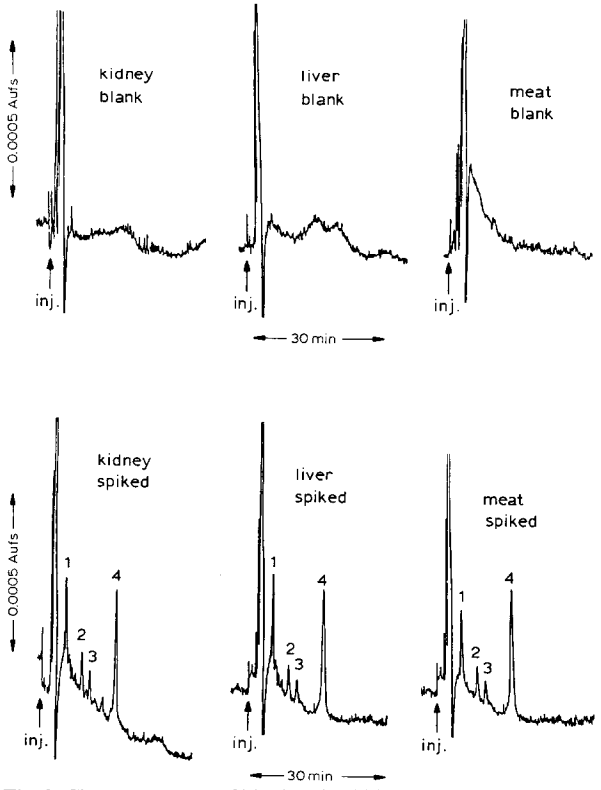

Fig. 8. Chromatograms of blank swine kidney, liver and muscle samples and corresponding samples spiked with 1 μg/kg of (1) CBX, (2) N^4-monooxy-CBX, (3) N^1-monooxy-CBX and (4) 5 μg/kg of desoxy-CBX. Detection at 420 nm, 0.001 a.u.f.s. For other experimental conditions, see text.

TABLE III

VETERINARY DRUGS THAT DID NOT INTERFERE WITH THE DETERMINATION OF CBX, N^1-MONOOXY-CBX, N^4-MONOOXY-CBX AND DESOXY-CBX

Standard solutions corresponding to a tissue concentration of 100 μg/kg were injected into the HPLC-system.

Drug	*Drug*
Chloramphenicol	Nitrovin
Chlorotetracycline	Olaquindox
Clopidol	Oxytetracycline
Dapsone	Pyranteltartrate
Decoquinate	Robenidine
Dimetridazole	Ronidazole
Dinitolmide	Sulphadiazine
Doxycycline	Sulphanilamide
Ethopabate	Sulphadimethoxine
Fenbendazole	Sulphadoxine
Furnicozone	Sulphamerazine
Halofuginone	Sulphamethazine
Ipronidazole	Sulphamethoxazole
Methylbenzoquate	Sulphaquinoxaline
Nicarbazin	Tetracycline
Nifursol	Thiophanate
	Trimetoprim

nation of N^1- and N^4-monooxy-CBX. In further studies, we shall attempt to include the important group of nitrofurans in the method in order to obtain a comprehensive method capable of monitoring the presence of both CBX and its metabolites and nitrofurans in animal tissues.

CONCLUSIONS

A rapid, sensitive and selective method has been developed for the trace-level determination of carbadox and its monooxy and desoxy metabolites in swine muscle, liver and kidney tissue. A liquid–liquid extraction followed by a simple alumina–Florisil clean-up and partitioning with isooctane is combined with column-switching HPLC with the post-column addition of alkali to convert the analytes of interest into yellow compounds that can be detected at 420 nm. The method permits the determination of concentrations down to 1 μg/kg of CBX and its monooxy metabolites and down to 5 μg/kg of desoxy-CBX. Preliminary recovery experiments indicate a good precision with a mean recovery of about 85%. About 20 samples can be analysed per day. The method also seems applicable to other biological matrices.

Further research is planned to study the ruggedness of the method, the incorporation of nitrofuran drugs in the method and the confirmation of positive samples by fluorescence, diode-array UV–VIS and mass spectrometric detection.

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