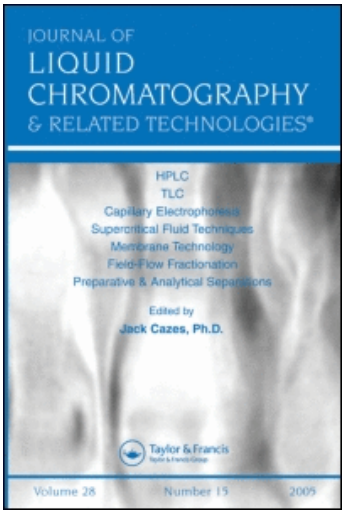

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SIMULTANEOUS DETERMINATION OF CHLORAMPHENICOL AND KETOPROFEN IN MEAT AND MILK AND CHLORAMPHENICOL IN EGG, HONEY, AND URINE USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

A liquid chromatographic-atmospheric pressure ionization ion spray method is described for the determination of chloramphenicol and ketoprofen in meat and milk, chloramphenicol in egg, honey, and urine. The samples were extracted with acetonitrile (acetone for urine), the organic layer was separated from water with chloroform and evaporated to dryness, and then purified using LMS solid phase extraction columns. The method is simple, requiring only small quantities of reagents and involves minimal manual work-up procedures.

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The lower limits of quantification were 1 ng/mL(g) for chloramphenicol and 5 ng/mL(g) for ketoprofen in milk and meat and 2 ng/g for chloramphenicol in egg and honey, and 3 ng/mL for chloramphenicol in urine.

INTRODUCTION

Chloramphenicol (CAP) is an effective broad-spectrum antibiotic. Because of its toxic properties(1, 2) CAP is not authorized for use in food-producing animals in Norway, the European Community countries, and U.S.A.(3). Ketoprofen (KTP) is a non-steroidal anti-inflammatory and analgesic drug used in veterinary practice.

These two drugs represent a potential health risk to consumers due to residues when used in food producing animals.

Several analytical methods for the determination of CAP in different biological materials, based on gas chromatography, gas chromathography mass-spectrometry, planar chromatography, liquid chromatography, pulse polarography, and immunoaffinity(4-10) have been published. Only one method for analysing KTP in meat has been described, based on liquid chromatography.(11) These methods are time-consuming, require large quantities of chemical reagents, involve extensive manual work-up procedure, or have poor sensitivity.

However, none of the published methods appear to be applicable for the simultaneous determination of CAP and KTP in meat and milk. Simultaneous determination of two or more drugs is desirable in drug residue control programmes because of the saving both in time and money.

The purpose of the present study was to develop a simple and specific method for the simultaneous determination of CAP and KTP in milk and muscle and CAP in egg, honey, and urine using LC-MS. The limits of quantification should at least meet the requirement of the MRL-values of CAP and KTP set by the EU Committee for Veterinary Medical Products.

EXPERIMENTAL

Materials and Reagents

Samples of milk, muscle, and urine from cow, poultry, egg, and honey were used. All chemicals and solvents were of analytical or HPLC grade. CAP and KTP were supplied by Sigma Co. (St. Louis, MO, USA). Stock solutions (1 mg/mL) of CAP and KTP were prepared in methanol and working standards

were prepared by diluting the stock solution with acetonitrile. The solutions were stored in a refrigerator (+4°C).

Solid phase extraction (SPE) columns, Bond Elut (1 cc/25 mg) LMS, were purchased from Varian (Harbor City, CA, USA).

Spin-X centrifuge filter units (0.22 μm, nylon type) from Costar (Cambridge, MA, USA) were also used.

Solution A, consisting of 0.5 M $Na_2HPO_4 \cdot 2H_2O$, was made by dissolving 44.5 g $Na_2HPO_4 \cdot 2H_2O$ in ca. 450 mL of water. The pH was then adjusted to 6.0 with 85% phosphoric acid, and the solution further diluted up to volume (0.5 L) with water, and the pH again adjusted to 6.0 with 85% phosphoric acid.

Chromatographic Conditions

The analyses were performed using a Perkin Elmer HPLC-MS system, consisting of a Series 200 quaternary pump and a Series 200 autosampler system (with 100% methanol as flushing liquid). The acquired data were entered into a Model 8500 Apple Power Macintosh and processed with either Multiview 1.4 or MacQuan 1.6 software packages (Perkin Elmer) for spectral information data processing and quantification data processing, respectively.

An API 100 LC-MS system (PE SCIEX) single quadruple mass spectrometer with a Turbo-Ion Spray Inlet for the API LC-MS system was employed for this study. The turbo probe of the instrument was maintained at 150°C and the flow/rate of air for the probe was 6 L/min. The LC/MS was set to collect multiple single-ion data in negative ion mode for ions at *m/z* 320.9 and 253 for CAP and KTP, respectively. The entrance electrode voltages were adjusted to provide the optimum overall intensities for the two molecular ions.

The optimal sensitivities for CAP and KTP were obtained with N2 nebulizer gas at 5 L/min, curtain gas at 10 L/min, and ion source at -4000V. For CAP the orifice was -20V and -10V for KTP. The ring was -330V and -300V for CAP and KTP, respectively, while the Quadrupole 0 was +10 for CAP and KTP.

A Merck column (Germany), Purospher Star RP-18 endcapped (stainless steel, 55 x 4 mm I.D. packed with 3 μm particles), was employed for measuring CAP and KTP. The column was connected to an A-318 precolumn filter on line with an A-102X frits (Upchurch Scientific, USA). The mobile phase for egg, meat, milk, and urine consisted of a mixture of two solutions, B and C (Tables 1 and 2). Solution B consisted of 0.15% formic acid in water (998.5 mL water +1.5 mL formic acid) while solution C was methanol.

The LC eluent was split post-column approximately 1:20 so that c. 50 μL flowed into the Ion-Spray ion source.

Table 1. Mobil Phase for Egg, Honey, Meat, and Milk Operating Conditions

Step	Time (min)	Flow (μL/min)	Solution B (%)	Solution C (%)
1	0.1	900	58	42
2	7	900	35	65
3	4	1300	58	42
4	1	900	58	42

Egg and Meat Sample Pretreatment

Seven millilitres acetonitrile or standard (the total volume should always be 7 mL) were added to 3 g egg or meat, weighed into a 50 mL centrifuge tube with screw cap (NUNC, Roskilde, Danmark). The mixture was homogenized for approximately 6 sec with an Ultra-Turrax TP 18/10 (Janke & Kunkel KG, Ika Werk, Staufen, Germany). After centrifugation for approximately 5 min. (5000 rpm), a 2.5 mL volume corresponding to 0.75 g sample was pipetted into a conical glass-stoppered centrifuge tube, and 4 mL $CHCL_3$ was added. The mixture was shaken vigorously for approximately 5 sec. After centrifugation for 2 min (3500 rpm), the upper layer (water) was discarded. The organic layer acetonitrile-chloroform was transferred to another glass-stoppered tube with a Pasteur pipette to avoid water residues.

The organic layer was evaporated to dryness under a stream of air using a Reacti-Therm heating module at 60°C and Reacti-Vap evaporating unit (Pierce, Rockford, IL, USA). The dry residue was dissolved in 100 μL methanol. Then 3 mL water and 0.5 mL solution A were added to the mixture and blended. The water-based sample was loaded into a conditioned LMS column.

Table 2. Mobil Phase for Urine Operating Conditions

Step	Time (min)	Flow (μL/min)	Solution A (%)	Solution B (%)
1	0.1	900	67	33
2	5	900	67	33
3	5	1000	37	63
4	5	1200	67	33
5	1	900	67	33

Milk and Urine Sample Pretreatment

Volumes of 0.5 mL CH_3CN or standard, 5 mL CH_3CN (acetone for urine) were added to 1 mL milk or urine. The mixture was shaken vigorously for approximately 10 sec. After centrifugation for 3 min (3500 rpm), the supernatant was transferred into a conical stoppered centrifuge tube, and 5 mL $CHCl_3$ was added. The mixture was shaken for 5 sec. After centrifugation for 2 min, the upper layer (water) was discarded. The organic layer acetonitrile/acetone-chloroform was transferred to another glass-stoppered tube with a Pasteur pipette to avoid water residues. Thereafter, the sample pretreatment was continued as described above for egg and meat.

Honey Sample Pretreatment

One millilitre water was added to 1 g honey. The sample was warmed up to 60°C for 5 min and the sample was vortex-mixed to the honey and was dissolved in the water. Five millilitres CH_3CN or standard was added. The mixture was shaken vigorously for 10 sec. After centrifugation for 3 min (3500 rpm) the supernatant was transferred to another glass-stoppered tube. One millilitre water and 5 mL $CHCl_3$ were added. The mixture was shaken vigorously for 6 sec. After centrifugation for 2 min, the upper layer (water) was discarded. The sample pretreatment was continued as described above for egg and meat.

Clean-Up SPE Column

The LMS column was conditioned with 1 mL methanol, followed by 2 x 1 mL water (column volumes). The aqueous extract was then put into the column, followed by 2 x 1 mL water and 0.3 mL water-methanol (90+10). Conditioning, application of the sample and washing took place under gravity flow (dropwise rate). Afterwards, the SPE column was suctioned to dryness for 10 sec. at a vacuum of -10 in. Hg. using a Vac Master system from International Sorbent Technology. The column was then eluted with 2 x 0.3 mL methanol at a vacuum of -3 in. Hg. After the eluting solvent had passed through, the column was suctioned to dryness for 5 sec. and to the eluate, 0.8 mL water was added.

The sample was mixed and filtered through a Spin-X centrifuge filter. Aliquots of 80 µL were injected into the LC-MS at intervals of 12 min for the determination of CAP and KTF in meat and milk. Aliquots of 80 µL were injected into the LC-MS at intervals of 12 min for the determination of CAP in egg and honey, and at intervals of 16 min 60 µL was injected for the determination of CAP in urine.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for CAP and KTP were determined by spiking cow muscle with standard solutions to yield 1, 2, 5, 10, 20, 30, and 50 ng/g for CAP and 5, 10, 20, 30, and 50 ng/g for KTP. Duplicate samples were used. The recovery rates were determined by comparing analyses of spiked muscle with those of standard solutions. The linearity of the standard curves for CAP and KTP in muscle was calculated using peak area measurements for CAP and peak height measurements for KTP.

RESULTS AND DISCUSSION

Chromatograms of drug-free meat samples and of corresponding samples spiked with CAP and KTP are shown in Figure 1. Chromatograms of drug-free milk samples and of corresponding samples spiked with KTP are shown in Figure 2. The standard curves were linear for the range of concentrations investigated (1 to 50 ng/g for CAP and from 5 to 50 ng/g for KTP). The corresponding correlation coefficients were 0.999 for CAP and KTP in muscle and milk, and 0.999 for CAP in urine. The recovery and repeatabilities for CAP and KTP are shown in Table 3.

Eight hundred μL water was added to the eluate from the SPE cartridge because 100% methanol will change the baseline resolution. In addition, large injection volumes of eluate broaden the peak and reduce resolution. On the other hand, more water in the final extract (sample) permits a larger volume to be injected into the column.(12)

To compare the analyses of spiked muscle with those of standard solutions, the corresponding standards were diluted with water-methanol (c. 60% water and 40% organic solvent is in the standard sample).

The chromatographic system appeared to be efficient for the determination of CAP and KTP in milk and muscle, and for CAP in egg, honey, and urine.

A new purospher Star RP-18 endcapped column (cartridges) employed for measuring CAP and KTP in this work, was activated with 100% acetonitrile for 30 min with a flow-rate of 1 mL/min, because the cartridge can dry out during stocking and shipping.

In many laboratories, a stream of nitrogen is used to evaporate CAP or KTP samples to dryness. We compared nitrogen and air produced from a central air compressor for evaporating the samples for CAP and KTP. No differences were observed and air is much cheaper than nitrogen.

The detection limit of the assay was calculated to be three times the baseline noise from drug-free tissue. No interference was seen during analysis, when calibrating the curves, or when performing recovery studies. For KTP in milk, it

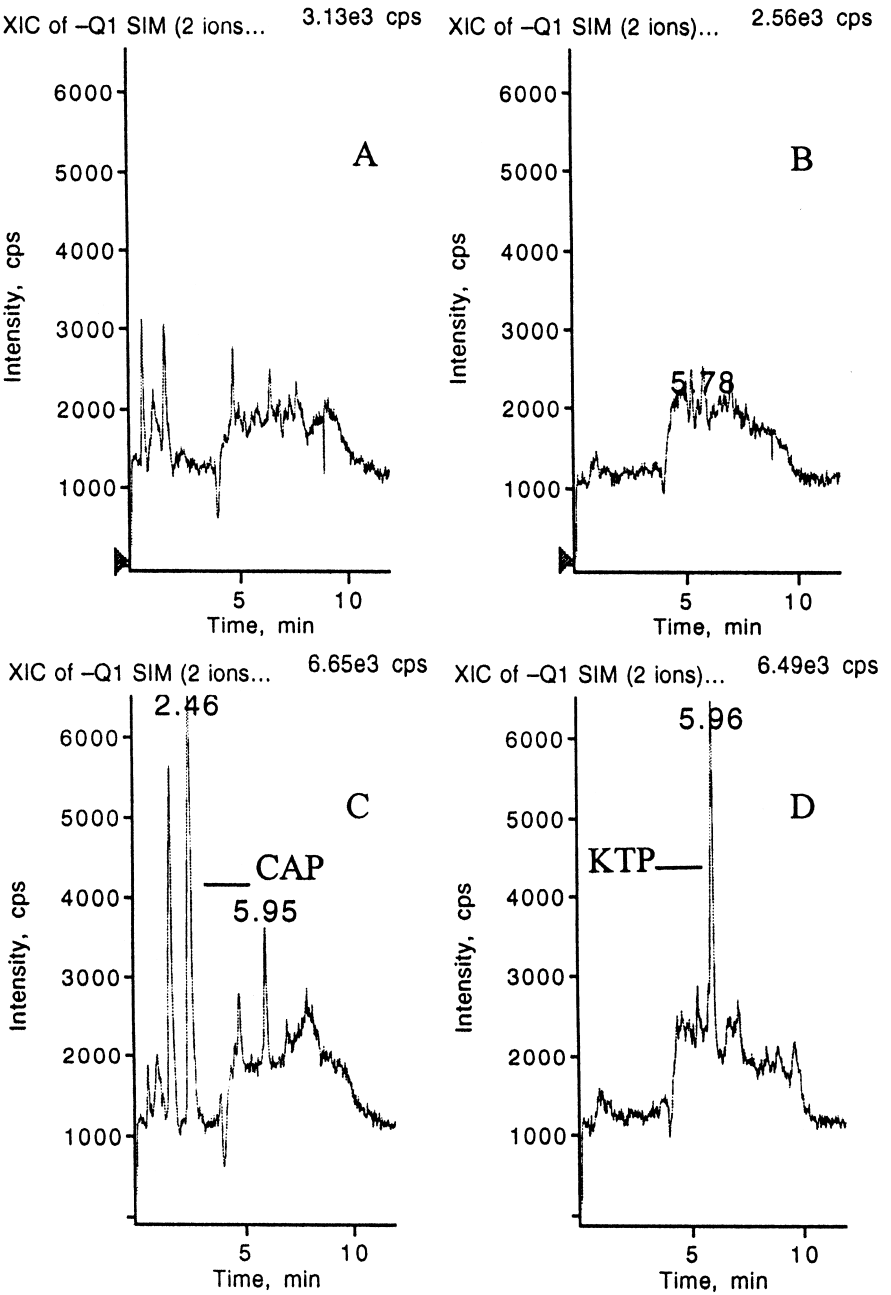

Figure 1. Chromatograms of extract from cow meat. **A:** Drug-free meat for CAP, **B:** drug-free meat for KTP, **C:** meat spiked with CAP (10 ng/g), **D:** meat spiked with KTP (10 ng/g).

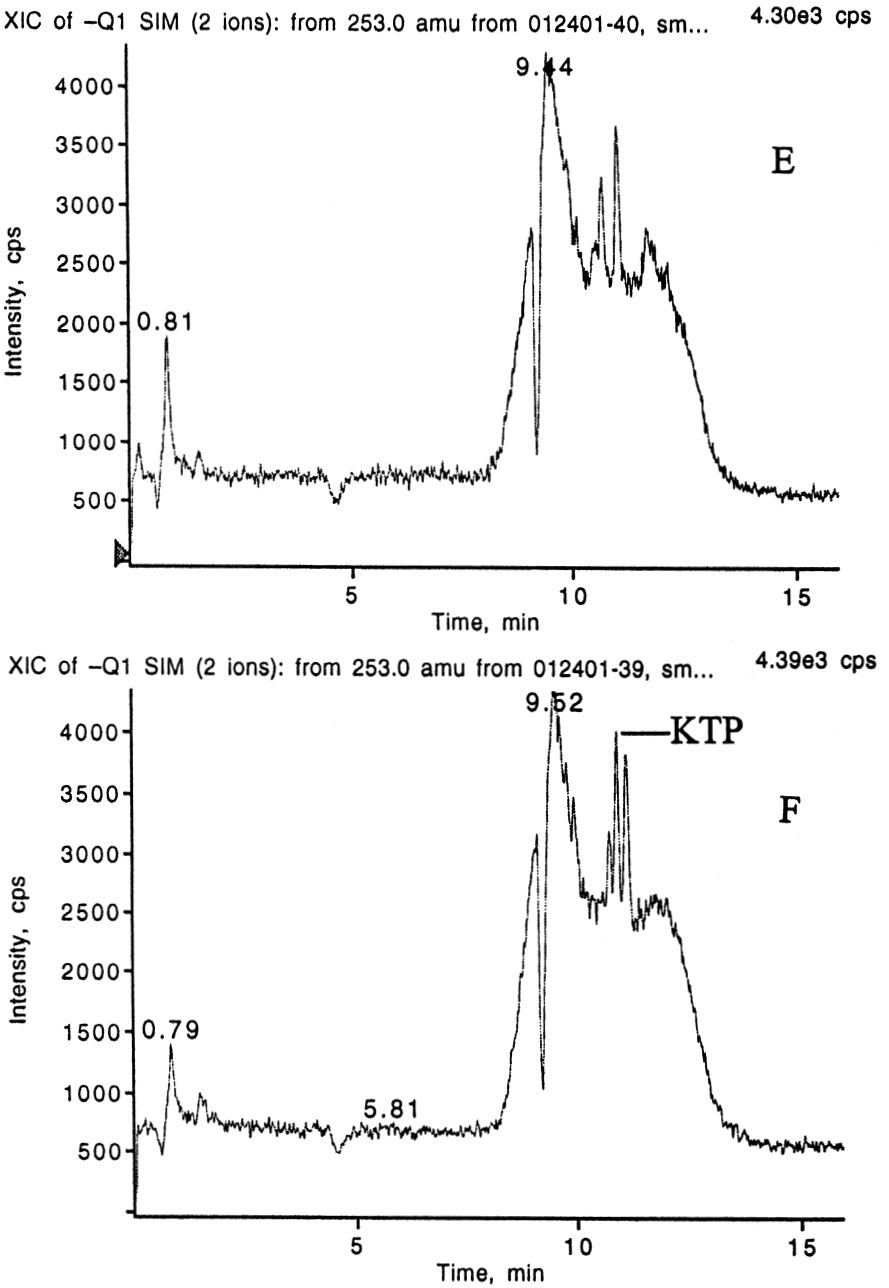

Figure 2. Chromatograms of extract from cow milk. **E:** Drug-free milk for KTP, **F:** milk spiked with KTP (5 ng/mL).

Table 3. Recovery and Repeatability for CAP and KTP from Spiked Samples of Cow Muscle

		Amount of Drug	CAP		KTP	
Sample	n	ng/g	SD %	Rec. %	SD %	Rec. %
Milk	8	10	1.1	98	0.4	98
	8	30	0.7	99	1.1	97
Muscle	8	5	0.5	95	2.9	95
	8	30	0.8	99	1.0	97
Urine	8	10	0.7	96		
	8	30	1.5	96		

SD= standard deviation. Rec.=recovery.

was necessary to use the same mobile phase operating conditions as for urine, because a interference was close to the KTP peak when the mobile phase operating conditions for meat were used.

The limits of detection were close to 1 ng/g for CAP in egg and honey, 0.5 ng/mL(g) for CAP in meat and milk, and 1.5 ng/mL for CAP in urine, and 2.5 ng/mL(g) for KTP in meat and milk. The limits of quantification were 2 ng/g for CAP in egg and honey, 1 ng/mL(g) in meat and milk, and 3 ng/mL for CAP in urine, and 5 ng/mL(g) for KTP in meat and milk.

The precision, recovery, and linearity of CAP in egg and honey were not validated in this study. It was not considered necessary to validate KTP in egg and honey. The method is poor for measuring KTP in urine. CAP in egg, honey, and milk show a similar baseline resolution to samples from meat.

The detection limit of the assay depends mainly on the sensitivity of the LC-MS. This in turn could be influenced by such factors as the position of the ion spray inlet (for CAP and KTP between 0.5 and 0.6 cm from orifice in horizontal position), the composition of the mobile phase, and the flow-rate of the mobile phase into the ion source.

The described assay offers a number of significant advantages compared to previously published methods for the detection and quantification of CAP and KTF. The detection limit is good. The extraction procedure is simple and only one extraction step is necessary. No derivatisation is required.

The advantage of the LC-MS technique lies in the combination of the separation capabilities of HPLC with the power of MS as an identification and confirmation method with high sensitivity, selectivity, and quantitative capability. Quantification using selected ion monitoring has high selectivity, sensitivity, and broad dynamic range. Thus, LC-MS seems to provide a better alternative than GC or HPLC.

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