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Determination of chloramphenicol in muscle, liver, kidney and urine of pigs by means of immunoaffinity chromatography and gas chromatography with electron-capture detection

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Abstract

A rapid and specific clean-up procedure based on immunoaffinity chromatography (IAC) with polyclonal antibodies for the gas chromatographic determination with electron-capture detection of chloramphenicol in pig muscle tissue, organs and urine is described. A commercially available IAC material was used for the analysis. A decrease in the capacity of the column after being used more than 100 times was observed. Mean recoveries were 69, 54, 62 and 95% for spiked pig muscle tissue, liver, kidney and urine, respectively. The limit of detection was 0.2 μg/kg for muscle tissue, 2.0 μg/kg for liver and kidney and 0.4 μg/kg for urine.

1. Introduction

Until the 1960s, chloramphenicol (CAP) was applied to different kinds of infectious diseases in human medicine, but today it is only used in small doses for particular indications because of toxic effects (aplastic anaemia). Further, it is very popular in veterinary medicine as a cheap and effective drug. In some countries, e.g., the USA, the application of CAP to food producing-animals is forbidden. In the European Community, the use of CAP is also forbidden according to the decision 1430/94/ECC [1].

Numerous clean-up methods have been published for use in the determination of CAP, but the use of an antigen–antibody reaction for clean-up [immunoaffinity chromatography (IAC)] is a relatively new method. In a few studies [2–4], IAC with monoclonal antibodies was used for the determination of CAP. There are several reviews that include residue analyses for CAP [5–7]. The principle of IAC is based on an antigen–antibody reaction. This principle employed in a clean-up analysis permits one specifically to concentrate an analyte such as CAP, by means of which clean extracts can be obtained.

In this work, the applicability of a commercially available polyclonal antibody for CAP coupled to an activated matrix (agarose) was investigated. The characterization of important quality parameters such as affinity, capacity and cross-reactivity of the material used was necessary [8]. The optimization of IAC was first carried out on CAP standard solutions and then with various matrices. In both instances the quality parameters were checked. As the detection method, the

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GC with electron-capture detection (ECD) was selected because very low limits of detection and determination are necessary after the banning of CAP [1]. The widely used HPLC methods have problems in the detection of CAP at very low μg/kg levels, in contrast to GC–MS and GC–ECD methods [5–7].

2. Experimental

2.1. Chemicals

Water was purified by demineralization. CAP was obtained from Sigma (St. Louis, MO, USA), *meta*-CAP and monochloro-CAP from Parke Davis (Freiburg, Germany), d_5-CAP from Promochem (Wesel, Germany), the IAC material from Laboratoire d'Hormonologie (Marloie, Belgium), hexadimethyldisilazane from Baker (Deventer, Netherlands), trimethylchlorosilane from Aldrich Europe and all other chemicals from Merck (Darmstadt, Germany).

A stock standard solution of CAP was prepared by dissolving 10 mg of CAP in 50 ml of 10% ethanol, and working standard solutions of 200 ng/ml (10% ethanol) and 20 ng/ml (10% ethanol) were subsequently prepared. Spiking solutions were prepared in the range 0.3–20 μg/kg tissue or organs or 0.5–90 μg/kg urine. Monochloro-CAP standard solutions were prepared in the same way.

Concentrated phosphate-buffered saline (PBS) was prepared by dissolving 80 g of NaCl, 14.33 g of $Na_2HPO_4 \cdot 2H_2O$, 2 g of KH_2PO_4, 2 g of KCl and 2 g of NaN_3 in 1 l of demineralized water. The PBS of pH 7.4 containing 0.14 *M* NaCl, 0.008 *M* $Na_2HPO_4 \cdot 2H_2O$, 0.002 *M* KH_2PO_4, 0.003 *M* KCl and 0.003 *M* NaN_3 was prepared by diluting the concentrated PBS 1:10 with demineralized water. The eluent used for the IAC procedure was 0.2 *M* glycine–0.5 *M* sodium chloride (pH 3).

2.2. Apparatus

The instruments used were an Ultra Turrax, a vortex mixer (Heidolph), a centrifuge (Sorvall), a sample concentrator with heating block, a Speed-Vac concentrator (Savant Instruments) and Econo-columns (connected with a three-way valve, No. 737-122, 10 cm × 0.7 cm I.D.; Bio-Rad Labs., Richmond, CA, USA) filled with 2 ml of immunosorbent.

The GC system was a Hewlett-Packard HP 5890 Series II gas chromatograph equipped with a ChemStation and electron-capture detector. Two different software systems were used, a Pascal- and a Windows-based system. A DB-5 (1 μm) GC column (25 m × 0.25 mm I.D.) from J&W was used. The carrier gas was argon–methane (95:5) at a flow-rate of 1 ml/min. The injection system was split–splitless (250°C), equipped with an autosampler; the injection volume was 2 μl and the column temperature was increased from 70°C at 15°C/min to 230°C (held for 10 min), then at 30°C/min to 280°C. The detector temperature was 300°C.

2.3. Sample preparation

A 3-g sample of muscle, kidney or liver or a 1-g sample of urine were weighed. All samples were spiked with CAP and monochloro-CAP as internal standard 2 h before extraction. For the recovery experiments, the samples were spiked with the internal standard after the clean-up.

Extraction procedure

To the samples 8 ml of acetonitrile–4% aqueous NaCl (1:1) were added, well mixed (Ultra Turrax), centrifuged for 10 min at 4000 *g* and the supernatant liquid was removed. Then, for further purification, 5 ml of *n*-hexane were added, mixed, centrifuged (1700 *g*) and the upper layer was discarded. A 5-ml volume of ethyl acetate (water-saturated) was added to the aqueous phase, mixed, centrifuged (1700 *g*) and the ethyl acetate extraction was repeated. The combined organic phase was evaporated to dryness under a gentle stream of nitrogen by using a heater (40°C). The dried residue was dissolved in 2 ml of 10% ethanol. If necessary, the samples were diluted, giving a final volume of 5–10 ml. For the urine preparation, hexane extraction was not necessary.

2.4. Immunoaffinity chromatography

The dissolved residue was carefully added to the IAC columns by using a Pasteur pipette without raising the gel, then the columns were kept at room temperature for 5 min. The total sample solution was drawn through the columns by gravity flow, the flow-rate being regulated at 0.5 ml/min with the three-way valve. The columns were washed with 10 ml of PBS buffer and 10 ml of water at a flow-rate of 1.5 ml/min. The antibody-bound CAP was eluted by gravity flow (1.5 ml/min) with 15 ml of ethanol or with 40 ml of glycine–NaCl (pH 3.0). After the antibody-mediated extraction, the columns could be regenerated for the next purification in the following way: the immunoaffinity columns were washed directly after elution with 10 ml of PBS buffer and 10 ml of water. The bottom ends were closed and ca. 10 ml of PBS buffer were added. The top ends were closed and the columns were carefully inverted several times. The homogeneous gel was then allowed to settle for 20 min, after which the columns were ready for re-use.

2.5. Extraction of the eluate

Glycine–NaCl eluate

A 15-ml volume of ethyl acetate (water-saturated) was added to the eluate, mixed, centrifuged (1700 *g*) and the ethyl acetate extraction was repeated. The combined organic phase was evaporated to dryness under a gentle stream of nitrogen by using a heater (40°C).

Ethanol eluate

The eluate was directly evaporated to dryness under a gentle stream of nitrogen by using a heater (40°C).

2.6. Derivatization

A 50-μl volume of the silylating agent chlorotrimethylsilane – hexamethyldisilazane – pyridine (1:3:9, v/v/v) was added to the dried residue, the solution was immediately evaporated under a gentle stream of nitrogen and the residue was dissolved immediately in 100 μl of *n*-hexane. A 2-μl volume was injected into the GC–ECD system.

3. Results

3.1. IAC parameter and characteristics

In Table 1, the most important parameters and characteristics of the applied IAC are given. In the following some aspects are explained in detail.

3.2. Capacity, recovery and re-use

The capacity of the columns was determined in the following way: 10 ml of a CAP standard solution (10 ng/ml) were added to the columns. The CAP-saturated columns were washed and eluted as described in Section 2.4.

The capacity of the columns was determined at the beginning and then after every fifth run (ethanol elution) or tenth run (glycine–NaCl elution). For this purpose, a calibration graph (range 10–80 ng) was applied and each calibration run was carried out three times. The internal standard was added after the IAC purification but before the derivatization. The dynamic column capacity was found to be 70 ± 2 ng of CAP per column as an average in each case (two and four columns, respectively) for each kind of elution, which means 35 ng CAP/ml gel, and the specific capacity is 38 ng CAP/mg per polyclonal antibody.

For the capacity determination, radioactively labelled material as proposed by the manufacturer was not chosen because working with radioactive material is restricted to special locations and causes problems of waste disposal.

The capacities of IAC material reported in the literature mostly show the same amount (30 ng/ml gel) [8], but sometimes a higher capacity of 5.04 μg/ml gel can be found [2–4]. It has to be proved for each individual case whether the capacity obtained is sufficient.

The use of 2 ml of gel for the described analyses depends on the small capacity of 1 ml of gel, which is insufficient for determining CAP in

Table 1
IAC parameters

Antibodies obtained from	Rabbit
Antibodies	IgG, purified on protein A
Kind of antibody	Polyclonal
Kind of gel used	Agarose gel
Protein concentration	0.924 mg IgG/ml gel
Capacity	70 ng per 2 ml gel
Affinity (under the chosen conditions)	$\sim 10^9$ l/mol
Recovery for CAP standard solutions:	95 ± 5%
Analysis temperature	~20°C (room temperature)
Reaction time at the beginning of IAC	5 min
Sample volume	5–10 ml
Flow-rate	Sample: ~0.5 min
	Washing and eluting: ~1.5 ml/min
Elution reagent and re-use (see Fig. 1)	15 ml of 70% ethanol (<20 times)
	40 ml of glycine–NaCl (pH 3.0) (>100 times)
Cross reactivity	With monochloro-CAP
	With d_5-CAP
Storage	At 4°C in presence of a buffer containing 0.01% NaN_3

different matrices, as shown in the following. Regarding re-use of the columns, two different results were obtained: on the one hand the capacity decreased dramatically after a few analyses with ethanol elution, but on the other no decrease was observed after using the immunoaffinity columns more than 100 times with glycine–NaCl elution.

The elution conditions have to be selected so that adequate reduction of the affinity constant is a reversible process and re-use is possible. With the two elution reagents used, two different results were obtained. On the one hand the two clean-ups used a different kind of desorption, i.e., they used different methods for breaking the antigen–antibody bond. As stated by the manufacturer of the IAC material, 70% ethanol causes a change in polarity. The reason for this is that the high ethanol concentration can denature the antibody, again leading to restricted re-use. Variations in the pH caused by the glycine–NaCl eluent resulted in improved re-use because of the essentially milder conditions.

On comparing the two elution reagents, glycine–NaCl is to be preferred. Although with ethanol elution a smaller volume is used, and although ethanol can be evaporated better and a second extraction after the elution is not necessary, which results in a shorter analysing time, eluents that can cause irreversible denaturation of the immobilized antibodies are undesirable because such eluents strongly restrict the repeated use of immunoaffinity columns. As milligram amounts of polyclonal antibodies are required for the preparation of one immunoaffinity column, the repeated use of these columns is a necessity for economic reasons [9]. When using monoclonal antibodies, economic factors do not play such a large role, because the production of monoclonal antibodies allows the use of relatively large amounts of antibodies and of disposable columns with moderate costs. The factors able to improve the elution procedure are not always compatible, which makes the choice of the final elution procedure very difficult.

The recovery for CAP standard solutions after glycine–NaCl elution was 95 ± 5% in the absolute range 1–70 ng. Hence no appreciable losses are observed when using IAC and losses in the matrix samples can be reduced by further purification steps.

3.3. *Affinity*

The affinity can be estimated with the following equation [8]:

$$\frac{V_e}{V_0} = 1 + k_a(q - p)$$

where k_a = affinity constant, V_l = elution volume of the substance, V_0 = void of the column, q = total concentration of binding sites, p = total (bound + free) concentration of ligand. With this equation an affinity constant for the polyclonal antibodies of $k_a \approx 10^9$ l/mol can be obtained under the above conditions. As the affinity constant of high-affinity antibodies varies from $1 \cdot 10^9$ to $1 \cdot 10^{13}$ l/mol, the polyclonal antibodies used can be classified as antibodies with moderate affinity. This estimation depends on the presumption that first V_0 (the volume of the stationary phase) is sufficiently characterized by the volume of the gel matrix (here 2 ml) and second the capacity is in accordance with the number of binding sites. These presumptions are only valid for the chosen conditions.

3.4. Cross-reactivity and specificity

The selectivity of IAC depends on the specificity of the immobilized antibodies used. If antibodies with a high specificity are used, cross-reactive and structurally analogous components can be eliminated. As a rule, this specificity is tested as the cross-reactivity of the antibodies, which is determined by obtaining dose–response curves by radioimmunoassay or enzyme-linked immunosorbent assay before they are bound to the activated gel matrix. Because in the described case the antibodies were already bound to the activated matrix, it was possible to test only chemical structurally analogous compounds of the CAP molecule with regard to their binding ability. Only such substances which can be used as internal standards were chosen. The following substances were tested: *meta*-CAP, monochloro-CAP, thiamphenicol and d_5-CAP. In each case 35 or 70 ng of the test substance were added to the IAC material, which correspond to half of and the total capacity of the IAC columns, respectively. Subsequently the amount of d_5-CAP could be determined by GC–MS and the other substances by GC–ECD. For each substance a calibration graph was applied in the range 10–80 ng, determining each point three times. CAP was added after clean-up before derivatization, and used as an internal standard.

Thiamphenicol and *meta*-CAP showed no signals under these conditions. For monochloro-CAP and d_5-CAP a capacity of 70 ± 2 ng could be determined again as for CAP. Because neither thiamphenicol nor *meta*-CAP could be determined after IAC and GC–ECD, in contrast to monochloro-CAP and d_5-CAP, it can be deduced in general that the *para*-nitrophenyl group of the chloramphenicol is specifically recognized by the polyclonal antibody. However, because of the limited availability of the analyte substances, which differ only in the position of the nitrophenyl group and the lack of this group, and because no information was given by the manufacturer on the binding of the antibodies to the activated matrix, it is not possible to draw conclusions about the influence of the acyl side-chain on the possible cross-reactions; in particular, some CAP metabolites could be possible cross-reactants. The antibodies might be conjugated with the acyl side-chain to the matrix, so that this part of the molecule is not responsible for the specificity of the antibody.

3.5. Matrix analysis

Muscle, liver, kidney and urine from pigs were used as matrices for this analysis. These matrices were spiked with CAP in a concentration range between the limit of detection for each material and 30 μg/kg. Most analyses were carried out on muscle, because muscle is the most important matrix for CAP residues. The analysis of organs and urine, however, indicates the useful applicability of the described method to more complicated matrices. The recoveries were determined by adding samples spiked with CAP to the IAC column without an internal standard. After elution and ethyl acetate extraction of the analyte, an internal standard was added to monitor the application of the samples, e.g., injection into the GC system. After a calibration graph had been prepared, the recovery was calculated from the different peak-area ratios between CAP and the internal standard.

Table 2
Recoveries

Sample	Average (%)	Standard deviation (%)	C.V. (%)	Concentration range (μg/kg)	n
Muscle	68.7	4.8	6.9	3–20	30
Liver	54.3	2.2	4.0	3–20	18
Kidney	61.7	2.9	4.7	3–20	18
Urine	95.9	7.9	8.2	3–90	18

For the determination of the limits of detection and determination, the internal standard was added to the samples at the same time as the CAP standard solution, i.e., before the IAC. The calculation of statistical parameters was carried out by using the following equations [10,11]:

limit of detection

$$= 2\frac{S_y t}{a_1}\sqrt{\frac{1}{N} + 1 + \frac{(y_c - \bar{y})^2}{a_1^2 \sum (x_i - \bar{x})^2}}$$

limit of determination

$$= \frac{y_h - a_0}{a_1} + \frac{S_y t}{a_1}\sqrt{\frac{1}{N} + 1 + \frac{(y_h - \bar{y})^2}{a_1^2 \sum (x_i - \bar{x})^2}}$$

where S_y = residual standard deviation, t = Student's factor, a_1 = slope, a_0 = intercept, N = sample size, $\bar{y}$ = average of all y_i, $\bar{x}$ = average of all x_i, y_c = upper limit of confidence at $x = 0$ and y_h = upper limit of confidence at $x = x_c$.

The results obtained are presented in Tables 2 and 3. In Figs. 1 and 2, chromatograms are shown as examples for 20 μg/kg of CAP in liver and 4 μg/kg of CAP in kidney, respectively.

Table 3
Limits of detection and determination

Sample	Limit of detection (μg/kg)	Limit of determination (μg/kg)	Concentration range (μg/kg)	n
Muscle	0.2	0.3	0.3–4	24
Liver	2.0	3.0	3–7	10
Kidney	2.0	3.0	3–7	10
Urine	0.4	0.6	0.5–5	10

4. Discussion

In general, the samples analysed by IAC should be in the form of an aqueous solution because organic solvents can interfere with the antibody–antigen interaction [2–4,9]. However, a protein precipitation is recommended for the determination of CAP, especially in tissues. To obtain good distribution ratios in the subsequent extraction steps the use of organic solvents is necessary [12]. The very good applicability of an

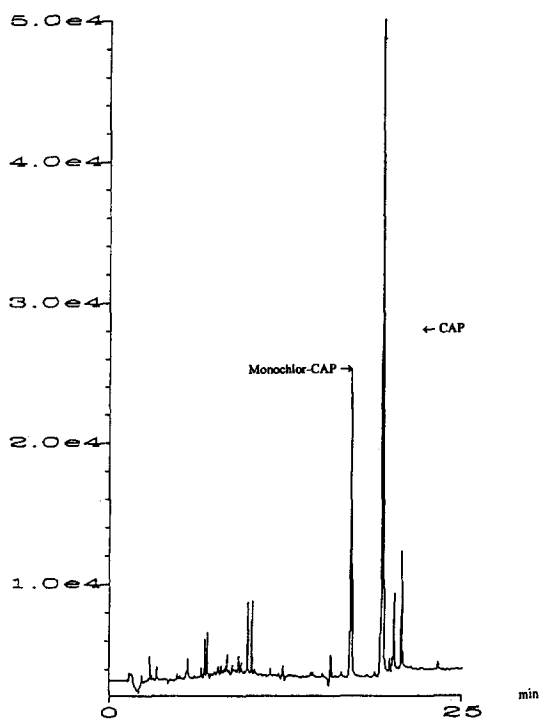

Fig. 1. Chromatogram of CAP in liver (20 μg/kg of CAP and 10 μg/kg of monochloro-CAP).

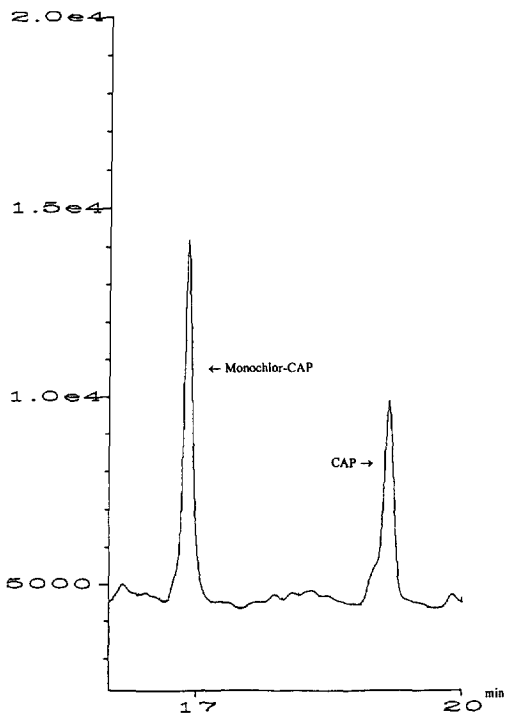

Fig. 2. Chromatogram of CAP in kidney (4 μg/kg of CAP and 6.7 μg/kg of monochloro-CAP).

acetonitrile–water mixture for the precipitation, hexane for the remove of lipophilic substances and ethyl acetate as a good extraction solution was shown previously [12]. The previously described extraction with water and subsequent filtration did not lead to satisfactory results because the background in the ECD chromatograms was too high [2–4]. Because of the possible interference of organic solvents in IAC, the last ethyl acetate extract had to be evaporated and the residue was dissolved in 10% ethanol. The addition of a small amount of ethanol to the residue did not affect the IAC material and was found to have a beneficial effect on the recovery. The determined recoveries of ca. 69% for muscle, 54% for liver, 62% for kidney and 95% for urine correspond to the data in the literature.

The decrease in recovery compared with the recovery of 95% obtained for CAP standard solutions can be explained by the losses in the first extraction before the IAC. An extraction was also carried out with ethyl acetate after IAC of the standard solutions, where the loss was very small. Further losses can be explained by non-separated matrix components, which can influence the binding of the antibodies by blocking the active sites.

The small coefficients of variation of the recoveries indicate the good repeatability of this method. No dependence of the recovery on the amount of CAP added was observed in the described ranges for each matrix.

The calibration graph procedure used for the calculation of the limits of detection and determination shows some important advantages in contrast to the generally used blank value procedure [10,11]: by using this calibration graph procedure, precise and exactly determined limits of detection and determination could be obtained for the different matrices. By analysing matrices from different animals (not done in this work), higher limits of detection and determination would be expected.

5. Conclusion

The described IAC procedure as a clean-up technique is very well suited to the determination of CAP. In combination with GC–ECD it is possible to detect very small amounts of CAP both in a simple matrix such as urine and also in more complicated matrices such as organs and muscle.

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