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Automated high-performance liquid chromatographic determination of chloramphenicol in milk and swine muscle tissue using on-line immunoaffinity sample clean-up

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ABSTRACT

An on-line high-performance liquid immunoaffinity chromatographic (HPLIAC) system for the direct determination of chloramphenicol in milk and swine muscle tissue is described. The system consisted of a dual-column system in which an HPLIAC column was directly coupled to an RP-8 high-performance liquid chromatographic column. Skimmed and deproteinated milk or aqueous muscle tissue extract was directly injected into the HPLIAC column. After a washing step with phosphate-buffered saline, chloramphenicol was desorbed by a glycine–NaCl buffer (pH 2.8) and directly concentrated on the RP-8 column. Next, chromatography was carried out using acetonitrile–sodium acetate buffer as the mobile phase. Chloramphenicol was detected at 280 nm. Mean recoveries from spiked raw milk were 70 ± 2% (1–50 μg/kg) and from spiked swine muscle tissue 64 ± 2% (10–50 μg/kg). The calibration curves were linear in the range 1–200 μg/kg spiking levels. Limits of determination were 1 μg/kg for milk and 10 μg/kg for muscle tissue.

INTRODUCTION

Inspection of food of animal origin for chloramphenicol (CAP) residues requires the availability of both qualitative and quantitative methods at or below the 10 μg/kg level. For this purpose many methods have been already developed (ref. 1 and references therein). In recent years, considerable progress has been made with respect to rapid and easy sample preparation procedures using solid-phase extraction and immunoaffinity clean-up (IAC). In the latter procedure specific monoclonal antibodies were applied [2,3]. This was done in an off-line mode preceding the high-performance liquid chromatographic (HPLC) determination. However, IAC can also be applied in an on-line mode, as described for the determination of β-19-nortestosterone and its metabolite α-19-nortestosterone in calf urine [4] and tissues [5], clenbuterol in urine [6], α- and β-trenbolone in calf and cattle urine [7] and aflatoxin M1 in milk [8]. In all these procedures the analytes were concentrated on the IAC column, reconcentrated on a small preconcentration column and subsequently eluted into the HPLC system.

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The purpose of this study was to develop an automated HPLC method with on-line IAC for the determination of CAP in milk and meat at low levels. In this procedure the application of high-performance liquid immunoaffinity chromatography (HPLIAC) was investigated. This technique can be considered as a combination of IAC and HPLC, in which antibodies are immobilized to an activated phase of the HPLC column. In the literature this technique has mostly been applied for preparative purposes (*e.g.* refs. 9–11) but can also be used for analytical purposes, *e.g.* for cortisol in urine [12].

EXPERIMENTAL

Reagents and chemicals

Water was purified by demineralization (conductivity < 1 μS). CAP and glycine were from Sigma (St. Louis, MO, USA). Hydrogen chloride, disodium monohydrogenphosphate, potassium chloride and sodium acetate were from J.T. Baker (Phillipsburg, NJ, USA), and trichloroacetic acid (TCA), sodium chloride, sodium dihydrogenphosphate, potassium dihydrogenphosphate, tris(hydroxymethyl)aminomethane (Tris) from Merck (Darmstadt, Germany). Acetonitrile and methanol (both HPLC grade) were from Rathburn (Walkerburn, UK) and sodium azide from BDH (Poole, UK). Filter paper circles (S&S 595½, diameter 125 mm) were obtained from Schleicher and Schüll (Dassel, Germany). The Durapore membrane filters (0.22 μm, 13 mm diameter) were from Millipore (Bedford, MA, USA).

Concentrated phosphate-buffered saline (PBS), pH 7.4, contained 1.368 *M* NaCl, 0.015 *M* KH_2PO_4, 0.081 *M* $Na_2HPO_4 \cdot 2H_2O$, 0.027 *M* KCl and 0.031 *M* NaN_3. It was diluted 1:10 with demineralized water.

A CAP standard solution was prepared by dissolving 100 mg of CAP in 100 ml of methanol. Working standards for HPLC were prepared in the range 1–200 ng/ml by diluting the standard solution in PBS. Spiking solutions containing 0.1, 1 and 10 μg/ml CAP were prepared by diluting the standard solution with demineralized water. The mobile phase for HPLC was acetonitrile–0.01 *M* sodium acetate buffer, pH 5.4 (1:3, v/v).

The coupling buffer solution (pH 7.5) contained 0.2 *M* NaH_2PO_4 and 0.5 *M* NaCl. The deactivation buffer was 0.2 *M* Tris–HCl (pH 8). The desorption buffer (pH 2.8) contained 0.2 *M* glycine and 0.5 *M* NaCl. Monoclonal antibodies against CAP were prepared and purified as described earlier [13].

Apparatus

The instruments used were a Moulinette homogenizer (Moulinette, Gouda, Netherlands), a table centrifuge (Heraeus Model Labofuge GL, Karlsruhe, Germany), an Ultra Turrax (Janke and Kunkel, Staufen, Germany), a shaking apparatus (Janke and Kunkel, Model S50), a vortex mixer (Scientific Industries, Bohemia, NY, USA), a magnetic stirrer (Pt 800, Protherm, Etten-Leur, Netherlands), a Reacti-Vap evaporating unit, Model 18780, connected to a Reacti-Therm heating module, Model 18790 (Pierce, Rockford, IL, USA). For the preparation of the HPLIAC column, a SelectiSpher-10 50 mm × 5 mm I.D. stainless-steel column, packed with an activated tresyl support was used (Pierce). The analytical column was a 5-μm Chromspher C_8 glass column (100 mm × 3 mm I.D., Chrompack, Bergen op Zoom, Netherlands) with a guard column (10 mm × 2.1 mm I.D.) packed with reversed-phase material (Chrompack).

For the automated HPLC system, an injection valve (Rheodyne, Model 7125, Cotati, CA, USA) equipped with a 5-ml sample loop, two HPLC pumps (Pharmacia LKB, Model 2150, Bromma, Sweden), a valve-switching unit (Multiport Streamswitch, Spark Holland, Emmen, Netherlands) containing two six-port switching valves and a solvent-selector valve, a variable-wavelength detector (Pharmacia LKB) operated at 280 nm, and a SP4270 integrator (Spectra Physics, San Jose, CA, USA) were used.

The scheme of the automated HPLC system is shown in Fig. 1.

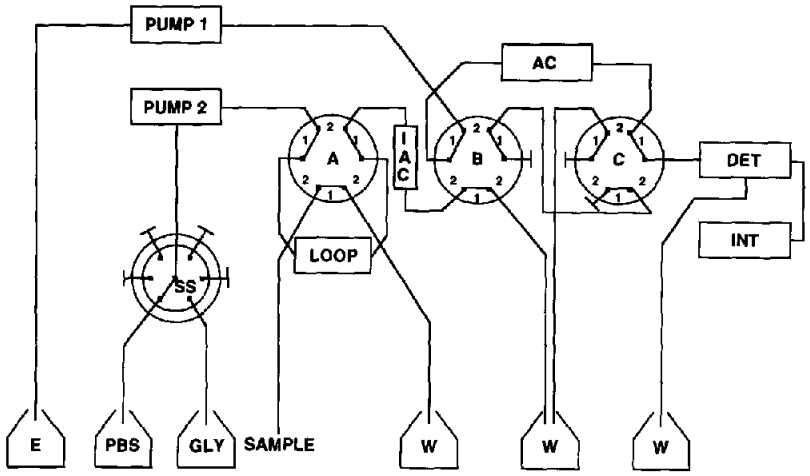

Fig. 1. Set-up of the automated HPLC system. AC = analytical column; IAC = immunoaffinity column; A = injection valve; B, C = switching valves; E = HPLC mobile phase; PBS = phosphate-buffered saline (pH 7.4); GLY = Gly–NaCl buffer (pH 2.8); W = waste; LOOP = 5-ml loop; DET = detector; INT = integrator; SS = solvent selector. The diagram shows the position when the sample is loaded into the HPLIAC column (Table I, 0–7 min).

Preparation of the HPLIAC column

The activated tresyl column was washed with 40 ml of demineralized water, followed by 45 ml of coupling buffer solution at a flow-rate of 1 ml/min using an HPLC pump. A 100-ml volume of a solution of 1 mg of monoclonal antibodies against CAP in 1 ml of coupling buffer was pumped through the column at a flow-rate of 1 ml/min, and the effluent was monitored at 280 nm. The column was washed subsequently with 35 ml of coupling buffer solution. As soon as the absorbance at 280 nm returned to the baseline, 25 ml of deactivation buffer was pumped through the column at a flow-rate of 1 ml/min in order to deactivate residual tresyl groups. When not in use, the HPLIAC column was stored in PBS at 4°C.

Samples

Fresh full-cream milk and swine muscle tissue obtained from untreated animals were used for the spiking studies. A real swine muscle tissue sample (sirloin) was obtained from a swine slaughtered 64 h after a single intramuscular injection of 60 mg of CAP per kg body weight in the neck [1]. Before analysis. as much visible fat and connective tissue as possible were removed from the pieces of lean meat.

Sample pretreatment

Milk. Approximately 10 g of homogenized milk were accurately weighed in a 20-ml polypropylene tube. The sample was centrifuged at 1300 *g* for 15 min. Fat (upper layer) was removed. Then 1 ml of a 15% TCA solution in water was added; the solution was thoroughly vortex-mixed and centrifuged at 2000 *g* for 10 min. To 7 ml of supernatant, 200 μl of a 2 *M* NaOH solution and 700 μl of concentrated PBS were added (final pH 7.2). The sample was filtered through filter paper, and 5 ml were injected into the HPLIAC system.

Muscle tissue. CAP was extracted from ground swine muscle tissue analogous to the procedure described earlier [2]. Approximately 10 g of ground muscle tissue were accurately weighed in a 125-ml polypropylene beaker. The sample was homogenized in 40 ml of demineralized water for 1 min using an Ultra Turrax. The homogenate was filtered through S&S filter paper and the filtrate was collected. The polypropylene beaker was rinsed with 20 ml of demineralized water.

TABLE I

PROGRAMME OF THE AUTOMATED HPLC SYSTEM

Time (min)		Event	Valve position			Solvent (pump 2)
Milk	Meat		A	B	C	
0–8	0–8	Load sample on HPLIAC column	1	1	1	PBS
8–35	8–25	Wash HPLIAC column	2	1	1	PBS
35–50	25–40	Transfer CAP from HPLIAC to analytical column	2	2	2	Gly–NaCl
49	39	Start integrator				
50–52	40–42	Wash HPLIAC column	2	1	1	Gly–NaCl
52–60	42–50	Condition HPLIAC column	2	1	1	PBS
50–60	40–50	Chromatography of CAP on analytical column	2	1	1	PBS
60	50	End run				

The wash liquid was also filtered. The volume of the total filtrate was adjusted to 55 ml with demineralized water, and 9 ml of the filtrate were added to 1 ml of concentrated PBS. The sample was filtered through the Durapore filter, and 5 ml were injected into the HPLIAC system.

HPLIAC procedure

Table I gives the schedule of the automated HPLC system. Pump 1 was used for the mobile phase, and the flow-rate was set at 0.6 ml/min. Pump 2 was used for PBS or glycine–NaCl, and the flow-rate was set at 0.7 ml/min. The samples were loaded onto the HPLIAC column for 8 min (pump 2, Fig. 1). Valve A was switched to position 2 to eliminate the effect of the 5-ml loop in the system. The column was washed with PBS: 27 min for milk and 17 min for muscle tissue. Then valves B and C were simultaneously switched to place both columns in series. Glycine–NaCl buffer was pumped through the HPLIAC column for 15 min to transfer CAP from the HPLIAC column to the analytical column. Next, valves B and C were switched again, and CAP was chromatographed on the C_8 column (pump 1, Fig. 1).

The HPLIAC column was flushed (pump 2, Fig. 1) with desorption buffer for 2 min to ensure complete removal of any CAP, and the column was reconditioned with PBS for 8 min. The total analysis time was 60 min for milk and 50 min for muscle tissue extracts.

RESULTS AND DISCUSSION

Development of the HPLIAC system

Immunoaffinity clean-up of crude samples or sample extracts prior to physicochemical determination is becoming increasingly popular for the determination of veterinary drugs in food of animal origin (ref. 14 and references therein). Depending on the specificity of the antibodies applied, this had led to very specific clean-up procedures as well as to procedures in which a single antibody has an affinity to more than one analyte. In most cases the antibodies are covalently bound to an activated support, such as agarose, trisacryl or Sepharose. These types of support, however, are unsuitable for automated sample treatment in which the IAC column, after loading of the analyte, is directly switched to the analytical column [4]. This is because of gel collapse as a result of the high pressure. Therefore, in the automated methods described hitherto, a preconcentration column, usually a C_{18}-bonded phase, has been introduced to which, for the desorption of the analyte, the IAC is switched in series prior to the HPLC determination.

Initial experiments as for developing an on-line automated immunoaffinity sample treatment for CAP in milk and muscle tissue were based on the same principle. A phenyl column proved to be suitable as the second precolumn for the capture of CAP from the glycine–NaCl solution used for

TABLE II

RECOVERY OF CHLORAMPHENICOL FROM SPIKED MILK AND MUSCLE TISSUE

Added (μg/kg)	Recovery (mean ± S.D., n = 6) (%)
Milk	
1	70 ± 5.7
20	67 ± 0.9
50	72 ± 0.9
Muscle tissue	
10	66 ± 2.1
20	62 ± 1.8
50	65 ± 1.0

the desorption of CAP from the IAC column. In the off-line procedure [3], it was already proved that, for desorption, a glycine–NaCl solution was essential in case the columns have to be used many times, although it was less effective than methanol, for example. The latter solvent, however, strongly restricted the re-use of the IAC column. A consequence of the use of the less efficient glycine–NaCl solution (elution volume *ca.* 20 ml) is that a small phenyl column (50 mm × 2.1 mm I.D.) causes breakthrough of CAP. On the other hand, a large phenyl column (100 mm × 3 mm I.D.) causes peak broadening and strong tailing of the CAP peak in the chromatogram.

For this reason, the application of HPLIAC was investigated. Monoclonal antibodies directed against CAP were coupled to an activated tresyl column as described. For desorption, a glycine–NaCl solution was used for reasons described before. In principle, the eluate can be directly monitored by a UV detector but, owing to the slowness of the desorption, significant peak broadening occurs. Therefore we chose a dual-column system in which the HPLIAC column was directly coupled to a C_8 analytical column. The eluate from the HPLIAC column was directly concentrated on the latter column, and chromatography was carried out with a mobile phase commonly used for HPLC analysis of CAP.

Spiking studies

Recovery experiments were carried out on raw milk at 1, 20 and 50 μg/kg spiking levels and on swine muscle tissue at 10, 20 and 50 μg/kg spiking levels. Each amount was added to six replicates.

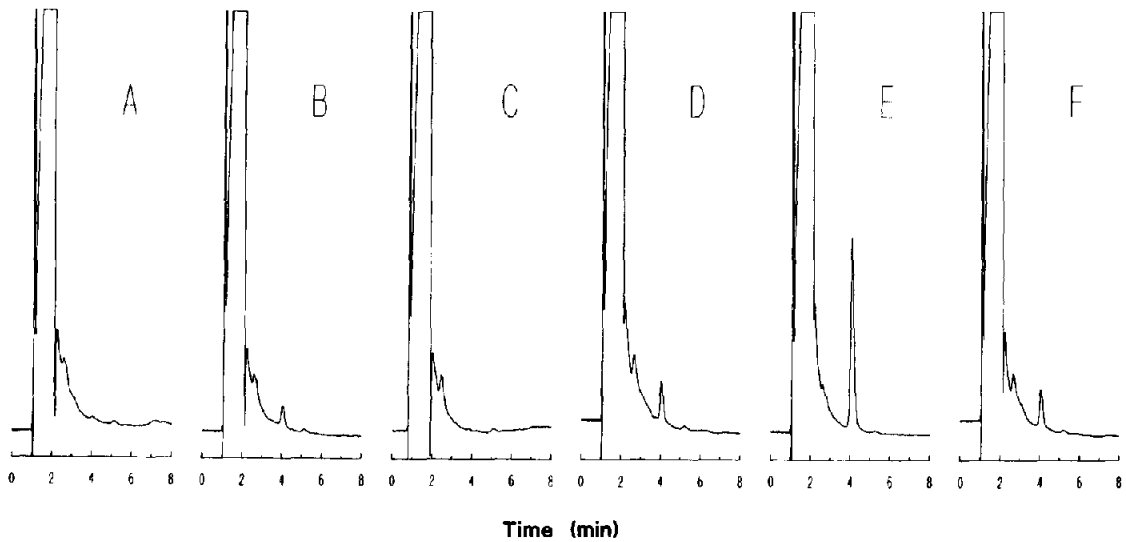

Fig. 2. Chromatograms of milk and meat samples: (A) blank milk sample; (B) spiked (1 μg/kg) milk sample; (C) blank swine muscle sample; (D) spiked (10 μg/kg) swine muscle sample; (E) real swine muscle sample (49 μg/kg); (F) standard solution of CAP (1 ng/ml). For chromatographic conditions see text. Absorbance range, 0.016 a.u.f.s. Time 0 corresponds to the start of the chromatography of CAP on the analytical column.

The samples, including the blank samples, were submitted to the procedure described above. The results are presented in Table II. The mean recoveries from spiked raw milk were 70 ± 2% and from spiked swine muscle tissue 64 ± 2%. Typical chromatograms are given in Fig. 2. Very clean chromatograms were obtained. No matrix interferences were observed, even at low levels. The total analysis time was 50 min for the meat samples and 60 min for the milk samples. The longer analysis time for milk is due to the fact that a longer washing period was necessary to eliminate interfering compounds from the matrix.

The system has been used for three months, during which about 150 samples were analysed, with no observed loss of analytical performance.

Real sample

The CAP content of the real swine muscle tissue sample was found to be 49 μg/kg. This result corresponds well with the results described earlier [1] for the off-line antibody-mediated clean-up procedure (*i.e.*, 45 μg/kg) and the solid-phase extraction procedure (*i.e.*, 49 μg/kg). A chromatogram of the real swine muscle tissue sample is also presented in Fig. 2.

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