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Simultaneous determination of β-lactam antibiotics in milk by ion-pair liquid chromatography

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Abstract

A simple and rapid ion-pairing liquid chromatographic method was developed for the simultaneous determination of five penicillins (PCs), ampicillin (AB-PC), benzylpenicillin (PC-G), cloxacillin (MCI-PC), dicloxacillin (MDI-PC) and nafcillin (NF-PC) in milk. These PCs are most frequently used for the treatment of mastitis of cows. These antibiotics were extracted with acetonitrile from milk and cleaned up by solid-phase extraction with a C_{18} cartridge. PCs were separated on a Kaseisorb LC ODS-300-5 column with a mobile phase (1 ml/min) of acetonitrile–methanol–0.05 *M* potassium dihydrogenphosphate (20:10:80, v/v/v) mixture containing 5 m*M* of sodium 1-decanesulfonate adjusted to pH 3.5 and UV detection at 210 nm. The average recoveries of five PCs from milk fortified at 0.5 and 1.0 μg/ml ($n=5$) were 79.8–89.4% with relative standard deviations ranging from 2.7 to 7.2%. The detection limit of PCs in milk were 0.03–0.05 μg/ml.

Keywords: Milk; Food analysis; Ion-pairing reagents; Antibiotics; Penicillins

1. Introduction

Mastitis is one of the most common diseases found in dairy cows. PCs, namely benzylpenicillin (PC-G), ampicillin (AB-PC), cloxacillin (MCI-PC), dicloxacillin (MDI-PC) and nafcillin (NF-PC) are mainly administered by various routes for the treatment of mastitis of cows in Japan.

In our country, the Cup Assay [1], the TTC Assay [2] and the Paper Disk Assay [3–5] have been used for detecting PCs residues in raw milk. The bioassay methods developed during 1960s, when PC-G was used exclusively in the treatment and control of bovine mastitis, are time consuming and lack the specificity. Because a variety of PCs are combined to use today, it is desirable to develop a simple and rapid method for simultaneous determination of PCs in raw milk which permits prompt residue monitoring.

Methods have been described for simultaneous determination of residual β-lactam antibiotics in milk by [6–15]. The methods described by Moats et al. [6–8], Munns et al. [9], Fletouris et al. [11] and Himei et al. [14] are not capable of detecting acidic PC groups and amphoteric AB-PC simultaneously. Also, a method by Straub et al. [13] involves mass spectrometry (MS) which limits the accessibility due to the cost. Sorensen et al. [15] describe a highly sensitive method, but requires derivatizing treatment of the samples.

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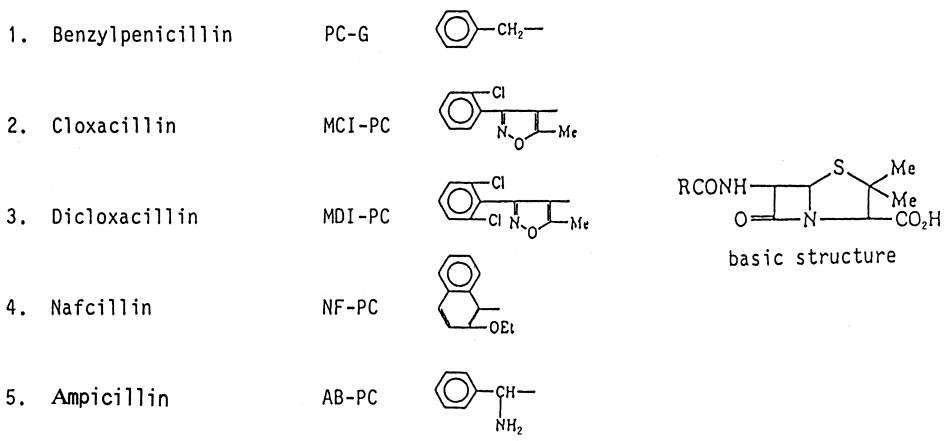

Fig. 1. Chemical structures of β-lactam antibiotics.

We developed a simple and rapid high-performance liquid chromatographic (HPLC) method with UV detection using ion-pairing reagent for a determination of PC-G, MCI-PC, MDI-PC, NF-PC and AB-PC (Fig. 1) in milk.

2. Experimental

2.1. Reagents

Ampicillin sodium salt (AB-PC), benzylpenicillin potassium salt (PC-G), cloxacillin sodium salt (MCI-PC), dicloxacillin sodium salt (MDI-PC) and nafcillin sodium salt (NF-PC) were obtained from Sigma (St. Louis, MO, USA). Individual stock solutions of 1 mg/ml were prepared by dissolving 10 mg of each penicillin in 10 ml distilled water. These stock solutions were diluted with mobile phase to give the desired concentration.

Sodium 1-octanesulfonate, sodium 1-nonanesulfonate, sodium 1-decanesulfonate, sodium 1-undecanesulfonate and sodium 1-dodecanesulfonate were purchased from Tokyo Kasei (Tokyo, Japan). All other reagents used were of analytical-reagent grade or better. Organic solvents were of HPLC grade.

2.2. Materials

Baker-10 solid-phase extraction (SPE) C_{18} cartridges 3 ml, 500 mg (J.T. Baker, Phillipsburg, NJ, USA) were conditioned with 3 ml methanol followed by 3 ml distilled water prior to use. Membrane filters used were Chromatodisc 13A, 0.45 μm (Kurashiki Textile, Osaka, Japan).

2.3. Apparatus

The HPLC system was purchased from Jasco (Tokyo, Japan) consisting of a Trirotar-VI pump, a Intelligent Sampler AS-950 and a UVIDEC-100-VI detector. Data plotting and analysis were done with a Chromatopac C-R3A (Shimadzu, Kyoto, Japan). Separation was performed on a Kaseisorb LC ODS-300-5, 5 μm, 250 mm×4.6 mm I.D. column (Tokyo Kasei). The mobile phase consisted of acetonitrile–methanol–0.05 *M* potassium dihydrogenphosphate (20:10:80, v/v/v) with 5 m*M* of sodium 1-decanesulfonate. The pH value of the mobile phase was adjusted to 3.5 with concentrated phosphoric acid. The column temperature was kept at 40°C and the flow-rate of mobile phase was 1.0 ml/min. UV detector was fixed at 210 nm.

A rotary vacuum evaporator EYELA N-1 (Tokyo Rikakikai, Tokyo, Japan) and a low-speed refrigerated centrifuge RL-601 (Tomy Seiko, Tokyo, Japan) were used in this study.

2.4. Sample preparation and extraction

A 20-ml volume of acetonitrile was added to 10-ml volume of milk in 50-ml glass centrifuge tube.

The mixture was vortexed for ca. 1 min and centrifuged at 1500 *g* for 10 min. The supernatant was transferred to a 100-ml round bottom flask and concentrated to 2–3 ml at reduced pressure to remove acetonitrile phase on rotary evaporator at 40°C. The concentrated extract was loaded into preconditioned Baker-10 C_{18} cartridge column and the entire sample was allowed to pass through. After the cartridge was dried for 3 min under vacuum and the analytes were eluted with 1.0 ml of methanol. The eluate was filtered through a 0.45-μm membrane filter and a 10-μl aliquot was injected onto the HPLC system for analysis.

3. Results and discussion

3.1. Analytical conditions

The chemical structures of PCs as shown in Fig. 1 indicate that PC-G, MCI-PC, MDI-PC and NF-PC are weakly acidic characters, and that AB-PC is amphoteric character. For the simultaneous determination of these PCs by HPLC, the authors examined the separation of these five PCs under the isocratic conditions with mobile phase consisting of ion-pairing reagent to form an ion-pair with AB-PC. Initial study was conducted on the mobile phase composition to obtain adequate separation among PC-G, MCI-PC, MDI-PC and NF-PC. In addition, five different alkylsulfonate salts composed of C_8 to C_{12} carbons were evaluated for optimal retention of AB-PC onto the column. By varying the proportions of the components of mobile phase containing acetonitrile–methanol–0.05 *M* potassium dihydrogenphosphate, the ratio 20:10:80 (v/v/v) provided an optimal separation of PCs. AB-PC was not retained on the column to any extent with addition of sodium 1-octanesulfonate (C_8) and sodium 1-nonanesulfonate (C_9) into the mobile phase. When sodium 1-decanesulfonate (C_{10}) was used, AB-PC eluted between PC-G and MCI-PC, and sodium 1-undecanesulfonate (C_{11}) and sodium 1-dodecanesulfonate (C_{12}) caused prolonged retention of AB-PC on the column. Therefore, sodium 1-decanesulfonate (C_{10}) was best suited ion-pairing reagent to retain AB-PC. A typical chromatogram of five PCs at a concentration 10 μg/ml is shown in Fig. 2.

In order to find the optimal wavelength for UV detection, the UV spectra of PCs standards dissolved in the mobile phase are measured as shown in Fig. 3. NF-PC exhibits the maximum absorption at 276 nm, 288 nm and 330 nm, but PC-G, MCI-PC, MDI-PC and AB-PC do not show any particular absorptions in UV region and hence, detection wavelength was fixed at 210 nm. Under the described conditions, the calibration curves for analyzed PCs were linear in the range of 0.05–0.3 μg.

3.2. Sample preparation

Various authors [6–9,11,14] reported the simultaneous determination of β-lactam antibiotic residues in milk, which included extraction procedures and yet insufficient sample clean-up. All these methods were unable to determine amphoteric AB-PC and other β-lactams simultaneously. Terada and Sakabe [10] extracted three PCs from milk using a SPE cartridge, because milk was pulled through directly into cartridge without deproteinization treatment, the separation of coextractives was not sufficient.

We performed the extraction of PCs from milk and deproteinization with two volumes of acetonitrile, and then acetonitrile was removed under the reduced pressure. Analytes in remaining aqueous solution was isolated by means of a C_{18} cartridge column. A matrix peak eluted during the initial 6 min on HPLC chromatogram and there were no interference peaks observed for PCs determination. A chromatogram obtained from milk fortified with all five PCs is shown in Fig. 4.

3.3. Recoveries

The recovery and the precision of the proposed method was determined, the results are summarized in Table 1. Milk samples were fortified at levels of 0.5 and 1.0 μg/ml, recoveries ($n=5$) ranged from 79.8–87.8% and 82.6–89.4%, respectively, with relative standard deviations (R.S.D.s) ranging from 4.6–7.2% and 2.7–5.3%, respectively. The limits of detection (signal-to-noise ratio 3:1) of PCs in milk were 0.03 μg/ml for AB-PC and PC-G, 0.05 μg/ml for MCI-PC, MDI-PC and NF-PC, respectively.

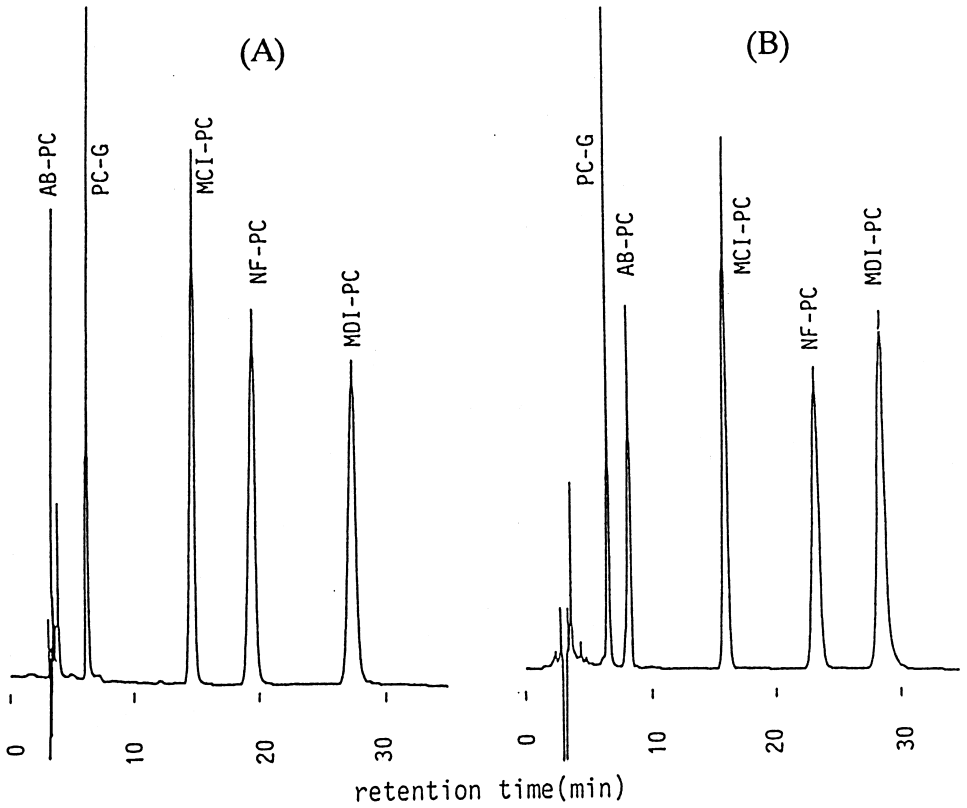

Fig. 2. Chromatograms of the mixed standard solution of five PCs (PC-G, AB-PC, MCI-PC, NF-PC and MDI-PC) concentration level at 10 μg/ml. Injection volume is 10 μl. Mobile phase: acetonitrile–methanol–0.05 *M* potassium dihydrogenphosphate (20:10:80, v/v/v) pH 3.5; (A) without ion-pairing reagent, (B) containing 5 mM sodium 1-decanesulfonate.

3.4. Affects of the other antibacterial drugs on chromatography

Although PCs are mostly administered for the treatment of bovine mastitis, it is sometimes used in conjunction with other antibacterial drugs. We investigated the affects of the other antibacterial drugs in milk on this method using 20 kinds of most commonly used antibacterial drugs [16] as shown in Table 2. Examining the UV spectra of these 20 antibacterial drugs in the mobile phase, dihydrostreptomycin, kanamycin, fradiomycin and polymyxine B exhibited no absorption within the UV region. Other 15 antibacterial drugs exhibited the absorption at detection wavelength 210 nm. These 15 drugs were fortified together with five PCs into milk and their retention time on HPLC was observed. Of these 15 antibacterial drugs, sulfadimidine (SMD), chloramphenicol (CP), oxytetracycline (OTC) and chlortetracycline (CTC) were remained on the column within the analysis time but others were all eluted out without any retention onto the column. As shown in chromatogram of Fig. 5, OTC and CTC are separated from PCs, but it was not possible to obtain a base-to-base separation of SDM, CP and PC-G. When PC-G was detected in milk samples, it was necessary to distinguish PC-G from SDM and CP by changing the detection wavelength where the maximum absorption of these drugs are observed, at 265 nm for SDM and at 275 nm for CP, respectively.

3.5. Analysis of raw milk sample

The method described in this paper was applied to the raw milk from a cow treated for mastitis with intramammary infusion drugs containing PC-G and

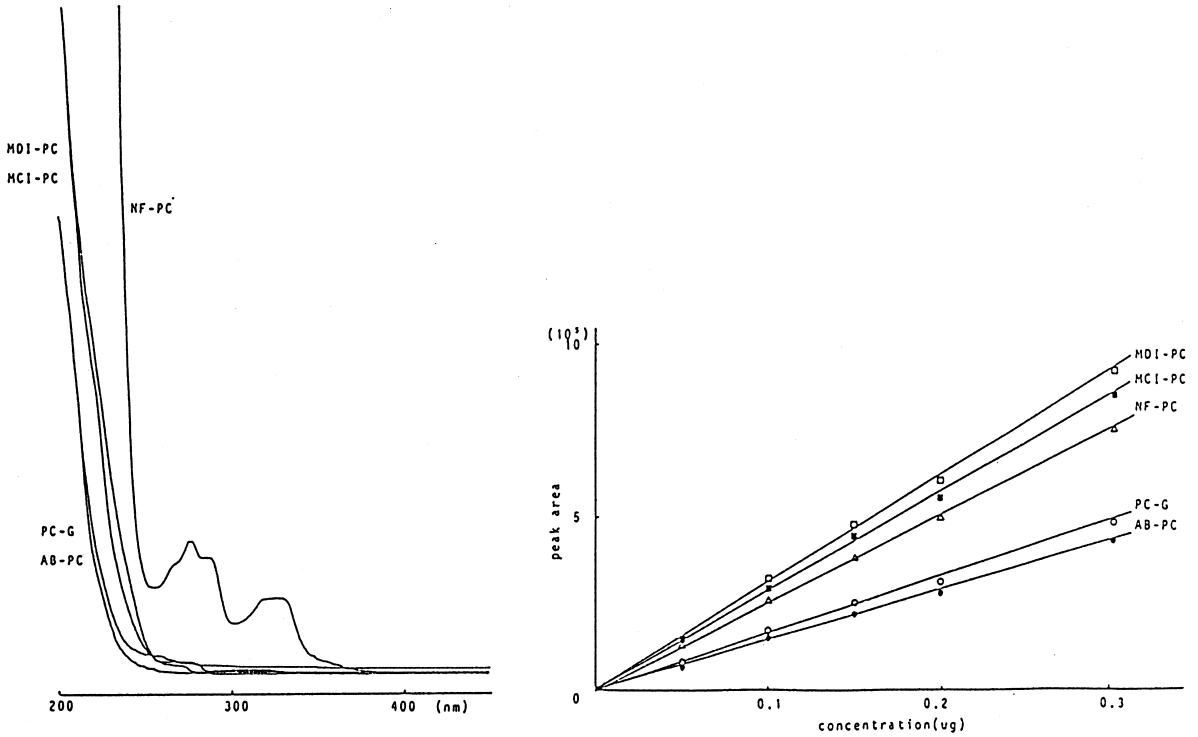

Fig. 3. UV spectra of PC-G, AB-PC, MCI-PC, NF-PC and MDI-PC. The standard calibration curves obtained from HPLC chromatograms for each penicillin within the range 0–0.3 μg.

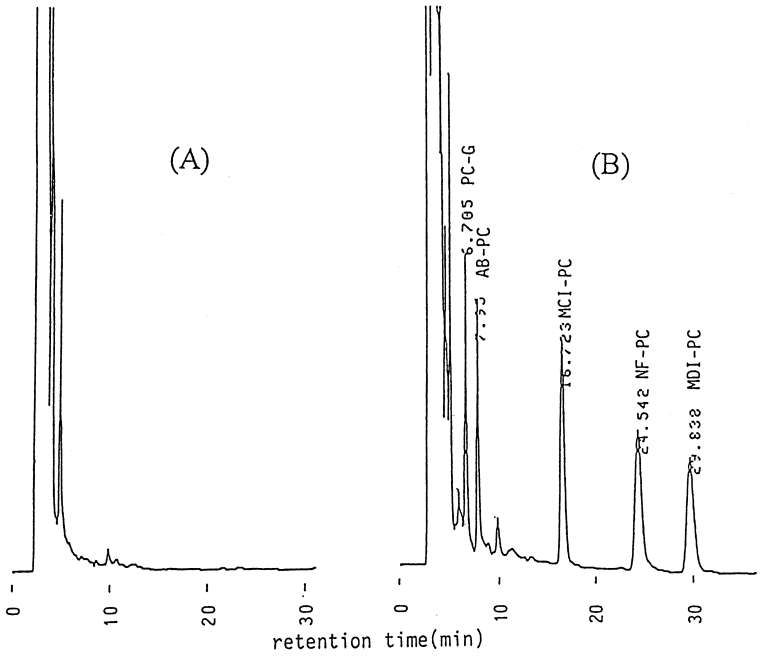

Fig. 4. Typical chromatograms of (A) blank milk and (B) milk fortified to a level of 0.5 μg/ml with penicillin.

Table 1
Recoveries of five PCs from fortified milk samples

Penicillin	Fortified (μg/ml)	Recovery (%) (mean±S.D.)	R.S.D. (%)
Ampicillin	0.5	84.2±4.8	5.7
	1.0	85.5±4.5	5.3
Penicillin G	0.5	79.8±3.7	4.6
	1.0	89.4±4.2	4.7
Cloxacillin	0.5	82.6±4.6	5.6
	1.0	84.9±2.3	2.7
Dicloxacillin	0.5	83.4±4.6	5.5
	1.0	82.6±2.3	2.8
Nafcillin	0.5	87.8±6.3	7.2
	1.0	89.3±4.6	5.2

n=5.

injection drugs containing AB-PC. The chromatogram obtained from the raw milk is shown in Fig. 6. PC-G and AB-PC were detected at the retention time corresponding to those of standards, with detection levels of 0.35 and 0.10 μg/g, respectively.

Table 2
List of antibiotics and synthetic antimicrobials commonly used for the treatment of mastitis other than penicillins

Group	Drug
Antibiotics	Dihydrostrepomycin
	Kanamycin
	Fradiomycin
	Oxytetracycline
	Chlortetracycline
	Erythromycin
	Kitasamycin
	Chloramphenicol
	Novobiocin
	Polymyxin B
Synthetic anibacterials	Homosulfamine
	Sulfadiazine
	Sulfadimethoxine
	Sulfisomidine
	Sulfisozole
	Sulfamerazine
	Sulfathiazole
	Furazolidone
	Nitrofurantoin
	Nitrofurazone

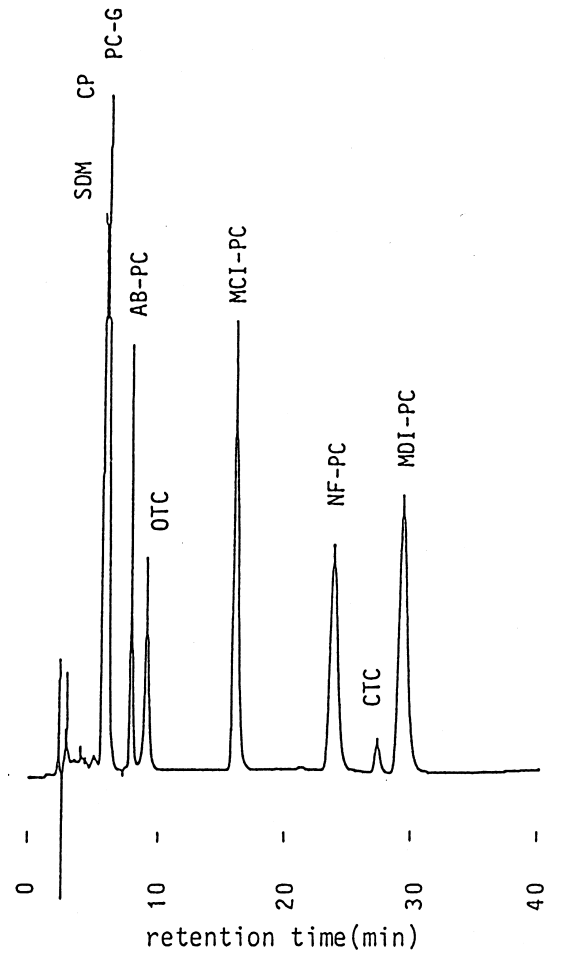

Fig. 5. Typical chromatogram of five PCs with sulfadimethoxine (SDM), chloramphenicol (CP), oxytetracycline (OTC) and chlortetracycline (CTC).

4. Conclusions

We described a simple and rapid HPLC method which allows the simultaneous detection of ampicillin, benzylpenicillin, cloxacillin, dicloxacillin and nafcillin in milk. Milk sample was deproteinized with acetonitrile and cleaned up through a C_{18} cartridge. The chromatographic technique involved an isocratic mobile phase with UV detection by reversed-phase ion-pairing method. The analysis time was ca. 30 min. Average of recoveries of five PCs were over 80% with R.S.D.s within 5%. The detection limit was 0.03–0.05 μg/ml. The proposed

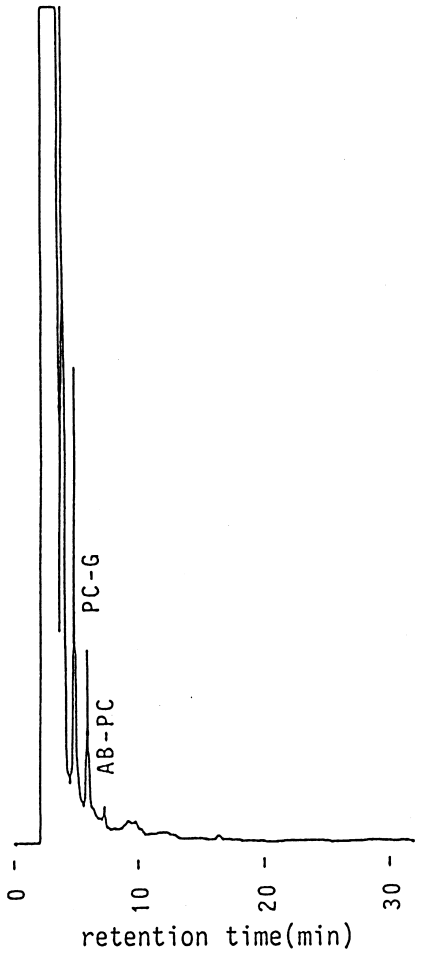

Fig. 6. Chromatogram of raw milk sample containing 0.35 μg/ml of PC-G and 0.10 μg/ml of AB-PC.

method may be applicable for analysis of PCs in milk.

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