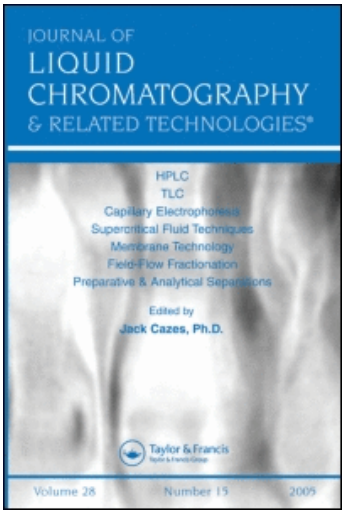


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# LIQUID CHROMATOGRAPHIC DETERMINATION/IDENTIFICATION OF RESIDUAL PENICILLIN G IN FOOD-PRODUCING ANIMAL TISSUES

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# LIQUID CHROMATOGRAPHIC DETERMINATION/IDENTIFICATION OF RESIDUAL PENICILLIN G IN FOOD-PRODUCING ANIMAL TISSUES

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## ABSTRACT

A simple and rapid high performance liquid chromatographic (HPLC) determination of residual penicillin G (benzylpenicillin, PCG) in cattle, pig, and chicken tissues (muscle, kidney, and liver) was developed. The sample preparation was performed by homogenizing with 0.1 M phosphate buffer (pH 6.5) followed by an Ultrafree®-MC/PL as a centrifugal ultrafiltration unit. For determination/identification of PCG, a Mightysil® RP-4GP column and a mobile phase of ethanol—0.1 M phosphate buffer (pH 6.5) (1:4, v/v) with a photodiode array detector was used. The average recoveries from spiked PCG (0.05, 0.1, 0.5, and 1.0 ppm) were in excess of 78.8% with coefficients of variation between 1.6 and 4.9%. The limit of detection was 0.04 ppm. The total time and solvent required for the analysis of one sample was <40 min and < 2 mL of ethanol, respectively.

*Key Words*: Penicillin G; HPLC; Ultrafiltration; Drug residues

## INTRODUCTION

Penicillin G (benzylpenicillin, PCG), a natural penicillin, is applied to the prevention or the treatment of diseases in food-producing animals and there is concern that residues of the compound may be retained in foods derived from treated animals. To prevent any health problems for consumers, the Food and Drug Administration (FDA), the Joint Expert Committee for Food Additives (JECFA) (Codex Alimentarium Commission in FAO/WHO), and the Japanese Ministry of Health and Welfare (JMHW) have set up PCG residue limits (FDA, tolerance; JECFA, maximum residue limit (MRL); and JMHW, standard residue limit) for PCG in livestock products. The common limit is 0.05 ppm (1–4).

The analytical method for residual PCG monitoring programs should be accurate, simple, economical in time and cost, and capable of detecting the residues below SRL/tolerance/MRL. Discharging the waste of toxic organic solvents is a severe problem with the world community. From the viewpoint of the toxicity of solvents and as to environmental effects, the method should avoid the use of toxic solvents and reagents.

For determination of PCG in biological samples, methods involving high performance liquid chromatography (HPLC) with various detection modes, i.e. direct ultraviolet (5–11), fluorescence (12), particle beam (13), and electrospray mass spectrometry (14,15). Recently, a precolumn reaction of PCG in various samples with 1,2,4-triazole-mercury (II) chloride solution has been described for HPLC analysis. The treatment technique was effective for the specific/sensitive detection of PCG, by which the derivatized PCG can be detected at 325 nm (16–19). However, these methods have any one of following problems: lack of sensitivity of confirmatory purpose at the SRL/tolerance/MRL; the extraction and clean-up involves numerous and varying analytical steps, which are time consuming and do not permit monitoring of a large number of samples; the use of toxic reagents, like mercury (II) chloride.

The present paper describes a simple and rapid method for HPLC determination of residual PCG in cattle, pigs, and chicken tissues, with no harm to humans and to the environment.

## EXPERIMENTAL

### Materials and Reagents

Edible tissues (muscle, kidney, and liver) of cattle, pigs, and chicken served as samples, and were stored in a refrigerator (−20°C) until analysis. Penicillin G (=benzylpenicillin, 1,595 U/mg) (PCG) was obtained from Wako Pure Chem. Ltd. (Osaka, Japan). Other chemicals were also obtained from Wako. Distilled water





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and ethanol were HPLC grade. $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$ were of analytical chemical grade.

Stock PCG standard solutions were prepared by accurately weighing 10 mg, dissolving it in 50 mL of 0.1 M phosphate buffer (pH 6.5) and diluting to the desired volume with the buffer. These solutions can be kept at 4°C for up to one week. The 0.1 M phosphate buffer (pH 6.5) for an extractant and the HPLC mobile phase was prepared as follows: Place 31.21 g $NaH_2PO_4 \cdot 2H_2O$ into 1 L volumetric flask and dissolve in distilled water. Dilute solution to volume with distilled water. Place 71.64 g $Na_2HPO_4 \cdot 12H_2O$ into another 1 L volumetric flask and dilute to volume with distilled water. Combine 685 mL 0.2 M $NaH_2PO_4 \cdot 2H_2O$ with 315 mL 0.2 M $Na_2HPO_4 \cdot 12H_2O$ in a 2 L flask. Dilute solution to 2 L volume with distilled water and mix. Adjust pH to 6.5 by adding, dropwise, either 0.1 M HCl or 0.1 M NaOH.



## Apparatus

The following apparatus were used in the sample preparation: Autohomogenizer, Ultra-Turrax® T25 (Iwaki Glass Co. Ltd., Funabashi City, Japan); Micro centrifuge, Biofuge® fresco (Kendo Lab. Products, Hanau, Germany).

As centrifugal ultrafilter units, three membrane types of Ultrafree®-MC series (nominal molecular weight limit (NMWL) = 5000, sample size ≤ 0.5 mL) were purchased from Millipore (Bedford, MA, USA): Ultrafree-MC/Biomax (Biomax™ high-flux polysulfone ultrafiltration membrane); −MC/PL (regenerated cellulose ultrafiltration membrane); and MC/PT (polysulfone ultrafiltration membrane).

HPLC analysis of the target compound was conducted using JASCO HPLC (Model PU-980 pump and DG-980-50 degasser) (Jasco Corp., Tokyo, Japan), equipped with SPD-M10A$_{VP}$ diode array detector (Shimadzu, Kyoto, Japan) interfaced with an FUJITSU FMV-5133D7 personal computer (Fujitsu, Tokyo, Japan). The separation was performed on a Mightysil® RP-4 GP (end-capped) (5 $\mu$m) column (4.6 × 250 mm) (Kanto Chem.), equipped with a guard column (4.6 × 5 mm) containing the same packing material, using a mixture of ethanol-0.1 M phosphate buffer (pH 6.5) (1:4, v/v) as the mobile phase at a flow-rate of 1.0 mL/min at ambient temperature. The injection volume was 100 $\mu$L.

## Calibration

Standard solutions of concentrations 0.01–0.5 $\mu$g/mL of PCG were prepared as the stock standard solutions. Volumes of 100 $\mu$L of these solutions were injected into the HPLC system. A calibration graph was obtained by measurement of peak areas.



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### Application of PCG to Ultrafree-MCs

A 0.5 mL of a standard solution containing 0.5 $\mu$g of PCG was put into the ultrafilter unit and centrifuged at 2000 $\times$ g for 5 min. The ultrafiltrate was determined by HPLC.

### Procedure

An accurately weighed 2.0 g minced sample was taken into a 10 mL test tube and homogenized with a autohomogenizer at 15,000 rpm for 5 min with 3.0 mL of 0.1 M phosphate buffer (pH 6.5). An 1.0 g of the homogenate was immediately transferred to a microcentrifuge tube and centrifuged at 10,000 $\times$ g for 5 min. A 0.5 mL of the supernatant liquid was put into Ultrafree-MC/PL and centrifuged at 2000 $\times$ g for 5 min. The ultrafiltrate was injected into the HPLC system.

### Recovery Test

The recoveries of PCG from blank samples spiked at 0.05, 0.1, 0.5, and 1.0 ppm were determined. These fortification levels were prepared by adding 20 $\mu$L of four standard solutions of PCG (5, 10, 50, and 100 $\mu$g/mL) to separated 2 g portions of the sample. Fortified samples were allowed to stand at 4°C for 12 h after the PCG standard addition and then mixed prior to workup.

## RESULTS AND DISCUSSION

### Sample Preparation

The present method could rapidly determine PCG in animal tissues, using HPLC without complex extraction and clean-up procedures; moreover, use of no organic solvents was also achieved. Namely, the sample preparation was performed by homogenizing with 0.1 M phosphate buffer (pH 6.5) followed by ultrafiltration using an Ultrafree®.

PCG degrades slowly when dissolved in water. A previous paper described that PCG had a peak of maximum stability at pH 6.5 and that stability dropped rapidly at higher and lower pHs (20). A 0.1 M phosphate buffer at pH 6.5 was used as an extraction solution and one of the mobile phase compositions for HPLC in this study. The extract did not form an emulsion that would hinder the recovery of a target compound.

***Table 1.*** Comparison on Recoveries[a] of PCG from Ultrafree-MCs[b]

| Type(Ultra-Filtration Membrane) | Recovery |
|---|---|
| Ultrafree-MC/Biomax | 69.8 (5.0) |
| Ultrafree-MC/PL | 98.2 (0.5) |
| Ultrafree-MC/PT | 72.9 (3.8) |

[a] Data are averages (%). $n = 5$; coefficients of variation in parentheses (%).
[b] A 0.5 mL of a standard solution containing 0.5 $\mu$g of PCG was put into the ultrafilter unit and centrifuged at 2000 $\times$ g for 5 min.



Table 1 presents the average recoveries of PCG standards from three membrane types of centrifugal ultrafilter unit when a standard solution containing 0.5 $\mu$g of PCG was applied to the ultrafilter units, respectively. An Ultrafree-MC/PL gave the best recovery (98.2%) and precision (CV = 0.5%) for PCG. This ultrafilter unit was able to deproteinize the extracted solution (a smaller sample, $\geq$0.5 mL) easily, in a short period (around 5 min), only with centrifuging. Ultrafree-MC/PL eliminates many steps and problems associated with classical clean-up techniques, e.g. emulsion, reduces recoveries, and consequently, increases clean-up yields. The technique also reduces analytical costs. The deproteinization technique gave good recoveries of PCG and prompt coagulation of protein in the tissue.

## HPLC Operating Conditions

Previous studies had reported that PCG in animal tissues could be acceptably determined by HPLC, by using the reversed-phase (RP) ($C_{18}$) column and a mixture of acetonitrile and phosphate buffer as the mobile phase (5,11). Acetonitrile and methanol are usually used in the mobile phase for RP-HPLC analyses of various compounds. These organic solvents are handled as the toxicity solvents (LD50 in orally in rats: 30–300 mg/kg B.W.). Considering the influence to the environment and to humans, an ethanol was used. A Mightysil® RP-4 GP ($C_4$) (end-capped) column with a mixture of ethanol–0.1 M phosphate buffer (pH 6.5) as the mobile phase, was examined regarding the separation: of PCG from the interfering peaks; the sharp peak obtained upon injection of equal amount of PCG. The chromatographic characteristics of RP columns can be very different because of variations in silica base, silanol group shield, alkyl chain length, or carbon content (21,22). Compared with $C_{18}$ column, the retention (=capacity



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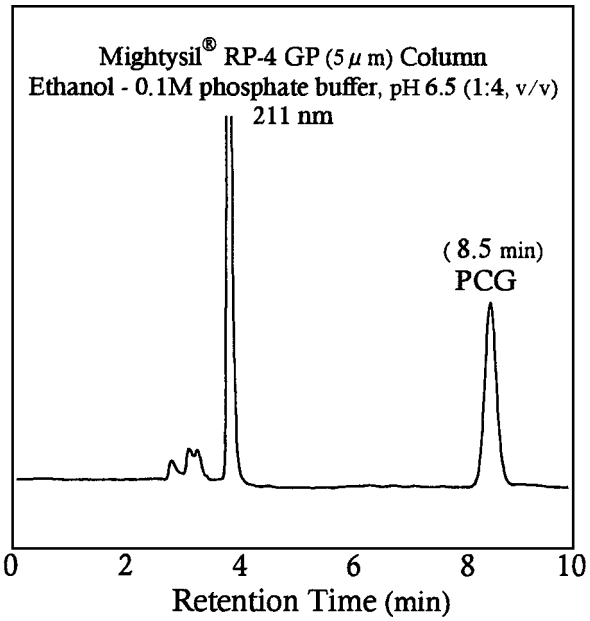


***Figure 1.*** HPLC chromatogram of PCG standard. A retention time of PCG (80 ng) = 8.5 min.

factor) on a $C_4$ column is generally weaker: the advantage of a $C_4$ column is that the column is able to analyze even the very polar compound, like PCG, by applying lower concentrations of organic solvent in the mobile phase, in a short time.

As expected, PCG was hardly eluted with 100% of the phosphate buffer. The best chromatogram with complete separation of PCG and interfering peaks and clear/short retention, was obtained using a mixture of ethanol–0.1 M phosphate buffer (1:4, v/v) as the mobile phase. A flow-rate of 1.0 mL/min gave a favorable retention time under the conditions examined over the range 0.8–1.5 mL/min. To reach maximum sensitivity, PCG's spectrum was measured by a photodiode array detector, and maximum absorbance was chosen: 211 nm was selected for PCG monitoring wavelength. Figure 1 shows a chromatogram of PCG standard obtained under the HPLC conditions. The sharp peak of target compound was detected symmetrically, without tailing. The retention time of PCG was 8.5 min. The solvent (only ethanol) consumption per sample was estimated to be $<2$ mL.

Figure 2 shows examples of typical HPLC traces of blank and spiked (PCG 0.1 ppm) cattle tissue samples obtained under the established procedure. The





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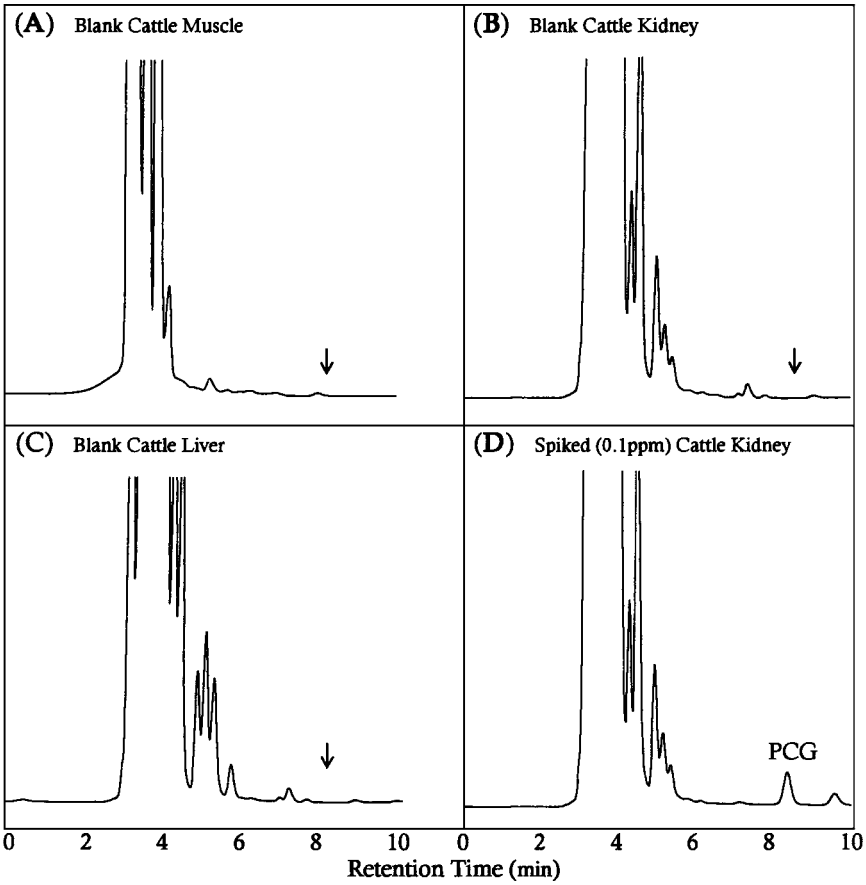


***Figure 2.*** HPLC chromatograms obtained from cattle tissue samples (photodiode array set at 211 nm). A retention time of PCG = 8.5 min. Arrows indicate the retention time of PCG.

resulting extracts were free from interfering compounds for detection and identification. This finding indicates that satisfactory purification could be archived by the present method. Similar chromatograms were obtained from pig and chicken tissue samples as shown in Figure 3. The present method made it unnecessary to use the gradient system to improve the separation.

With the proposed procedures, shorter analysis time and use of less organic solvent (no toxicity solvents and reagents) was achieved. Analytical time and solvent consumption were <40 min/sample and <2 mL of ethanol/sample, respectively.

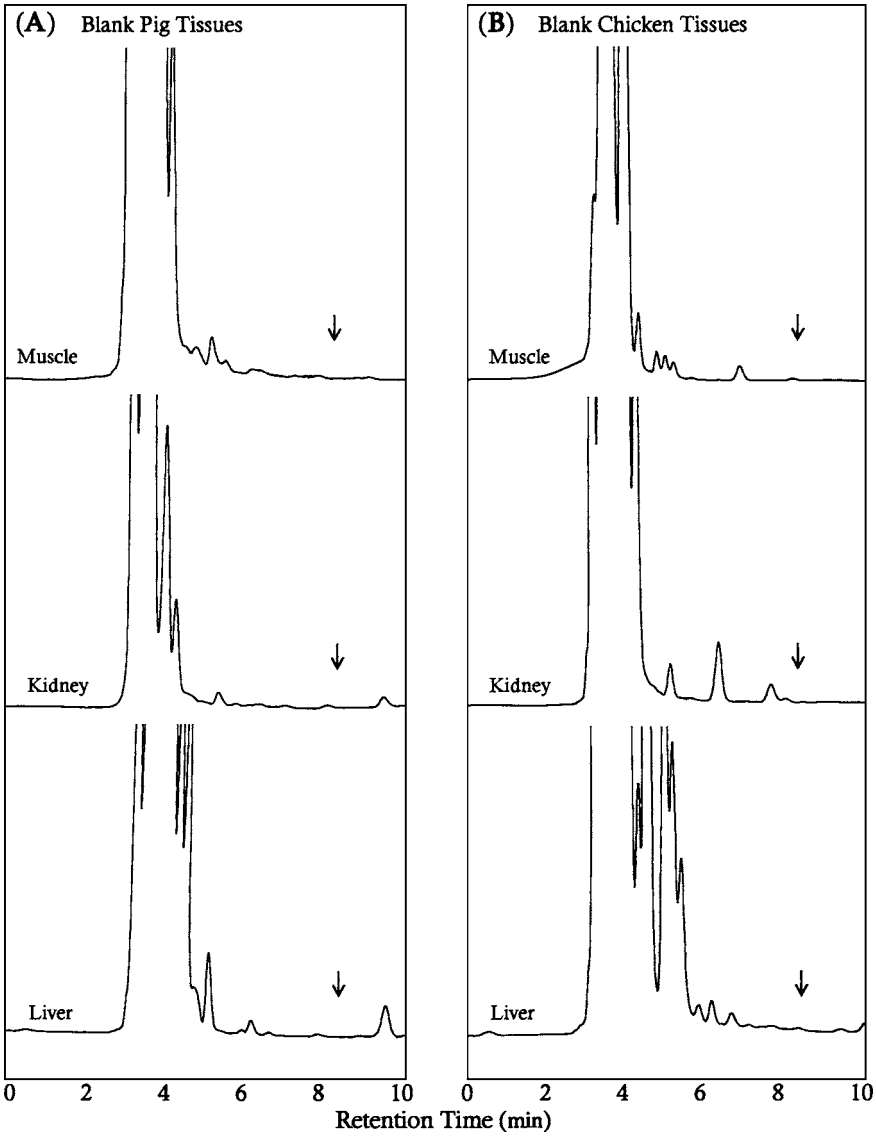


***Figure 3.*** HPLC chromatograms obtained from blank pig (A) and chicken (B) tissue samples (photodiode array set at 211 nm). Arrows indicate the retention time of PCG (*See* Figs. 1 or 2).

## Calibration

The calibration graph obtained by plotting peak area against amount, was linear over range 1–50 ng and passed through the origin. The correlation coefficient, 0.999, was highly significant statistically ($P < 0.01$). The minimum detectable amounts (peak-to-noise ratio >3) of PCG was 1 ng. The precision of the HPLC procedure was obtained from relative standard deviation of areas calculated for ten replicate injections of a 5 ng of PCG. The value was 0.8% for PCG.

## Recoveries and Identification

For three tissues obtained from three different animals, respectively, "the standard graph" was generated by plotting peak areas of fortified sample extracts for the concentration examined (0.05, 0.1, 0.5, and 1.0 ppm). Each graph was constructed from four points and each point represented the mean of the five injections. All of the correlation coefficients, between 0.997 and 0.999, were highly significant statistically ($P < 0.01$). These nine graphs, and the above graph of pure standards able to pool statistically, indicates that slopes of the nine standard graphs are similar to that of pure standards. The calibration can be carried out with the simplest procedure using pure standards.

Table 2 summarizes the average recoveries of PCG from cattle, pig, and chicken tissues at four different spiking levels (0.05, 0.1, 0.5, and 1.0 ppm). These results were satisfactory. The average recoveries were greater than 78.8% with coefficients of variation (CV) between 1.6 and 4.9%. A practical limit of detection which give a clearly discernible peak of PCG (signal-to-noise ratio >5) was 0.04 ppm. The value was below the SRL/tolerance/MRL (0.05 ppm). These findings indicate that the present method has good precision and is accurate.

Additionally, the "repetitive recovery test" using a homogenate (mixture of 2 g sample and 3 mL the buffer) obtained from a spiked tissue sample were done in triplicate. For example, the recoveries from a spiked (PCG 0.5 ppm) cattle muscle sample were 86.9, 87.7, and 89.2%, respectively. There was no difference among these three data. Similar results were obtained from the other tissues and animal tissues.

In HPLC analysis for residual drug monitoring, a photodiode array gives spectral information and is an easy method for the confirmation of the drug. HPLC combined with the diode array system proved to be able to detect a wide range of molecules and ensure identification of target compound. The retention time and spectrum provide strong evidence of its identity. PCG could be identified in the tissue sample with its retention time and absorption spectrum. The spectrum of PCG obtained from a sample is practically identical with that of





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***Table 2.*** Recoveries[a] of PCG from Cattle, Pig, and Chicken Tissues

| Spiked Sample (ppm) | Cattle | Pigs | Chickens |
|---|---|---|---|
| Muscle | | | |
| 0.05 | 81.3 (4.6) | 79.9 (4.2) | 80.9 (2.9) |
| 0.1 | 80.6 (3.1) | 86.1 (4.6) | 80.6 (3.8) |
| 0.5 | 88.1 (3.2) | 87.6 (1.6) | 88.8 (2.1) |
| 1.0 | 89.2 (2.9) | 87.2 (2.0) | 90.3 (2.4) |
| Kidney | | | |
| 0.05 | 80.2 (4.2) | 82.3 (3.1) | 86.4 (3.9) |
| 0.1 | 81.9 (3.9) | 86.4 (2.9) | 83.3 (2.6) |
| 0.5 | 84.9 (3.6) | 88.0 (3.5) | 82.5 (4.4) |
| 1.0 | 85.4 (1.8) | 86.6 (3.6) | 89.5 (1.7) |
| Liver | | | |
| 0.05 | 82.9 (4.0) | 81.1 (4.6) | 78.8 (4.9) |
| 0.1 | 79.9 (4.9) | 83.0 (3.0) | 84.0 (2.5) |
| 0.5 | 81.6 (2.3) | 88.5 (2.9) | 79.4 (4.0) |
| 1.0 | 82.3 (3.3) | 80.4 (2.6) | 82.2 (3.3) |

[a]Data are averages (%). $n = 5$; coefficients of variation in parentheses (%).

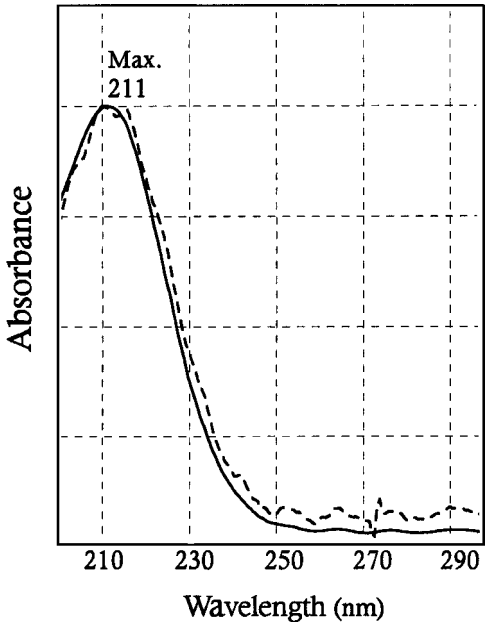


***Figure 4.*** Normal absorption spectra of peaks at 8.5 min for PCG in chromatograms (*See* Figures 1 and 2). PCG standard (solid line); spiked (PCG 0.1 ppm) cattle kidney extract (dashed line).





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the standard. Figure 4 gives a spectrum of the PCG peak of cattle kidney sample obtained with the photodiode array detector. Similar results were obtained from the other tissue samples. The present sample preparation permitted reliable confirmation.

### Monitoring Residue in Marketing Animal Tissues

Fifty different samples of marketing animal edible tissues that were circulated in Osaka City were analyzed by using the present method. No PCG was detected. There were no interfering peaks in the resulting chromatograms.



## CONCLUSION

A rapid and simple determination method of PCG in cattle, pig, and chicken tissues by using HPLC was developed. The proposed procedure is simple and easy, gives a shorter analysis time (total <40 min/sample), is more precise (CV $\geq$ 4.9% in the recovery test) and harmless (total solvent consumption <2 mL of ethanol/sample). Therefore, this procedure may be useful for the routine monitoring of residual PCG in muscle, kidney, and liver of cattle, pigs, and chickens.

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