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# FORMATION AND METABOLISM OF PUNGENT PRINCIPLE OF *CAPSICUM* FRUITS

## XV. MICRODETERMINATION OF CAPSAICIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A new, highly sensitive method for quantitative separation and determination of capsaicin and dihydrocapsaicin by high-performance liquid chromatography with electrochemical detection is elaborated. The method employs a Cosmosil 5Ph column eluted with 100 m*M* potassium dihydrogenphosphate containing 45% acetonitrile (pH 5.0) at a flow-rate of 1.0 ml/min, and an electrochemical detector (at a potential of +750 mV *versus* the Ag/AgCl electrode). The detection limits for both capsaicin and dihydrocapsaicin were 12 pg (39 fmol) at a signal-to-noise ratio of 3:1. By applying this method, the biological half-life of capsaicin in the rat was investigated.

## INTRODUCTION

Capsaicin (CAP) and dihydrocapsaicin (DC) are the main components of hot red pepper, constituting 90% or more of the total pungent principle[1,2]. Current interest in CAP and its analogues has been directed to its chronic and acute toxicity, its effects on the nervous system and its nutritional benefits. The actions of CAP and its analogues were reviewed by Virus and Gebhart[3], Nagy[4] and Buck and Burks[5]. Recently, we have shown that CAP and DC administered orally were absorbed into the gastrointestinal tract, and metabolized in rats[6].

High-performance liquid chromatographic (HPLC) methods with UV detection[2,7–10] and fluorescence detection[10–12] have been used to analyze CAP and its analogues. However, these methods have inadequate sensitivity to elucidate the nutritional and pharmacological effects of the pungency. Therefore, a new detector was required. A polarographic method for the detection of organic molecules has proved fairly sensitive; it is possible to determine concentrations of $10^{-7}$–$10^{-8}$ $M$[13,14]. In 1973, Kissinger *et al.*[15] reported the development of an electrochemical detector using carbon paste as the working electrode. With this detection method, they succeeded

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in determining biogenic samples such as catecholamines[16] and pterins[17]. Electrochemical detection (ED) is selective rather than versatile, which permits the ready elimination of electrochemically inactive concomitants or impurities contained in samples, reagents or the mobile phase. HPLC-ED was recently reviewed by Kissinger *et al.*[18] and Majors *et al.*[19]. In this paper, we describe a useful HPLC method with ED for the determination of CAP in rat plasma. This method is highly sensitive, simple and reproducible.

## EXPERIMENTAL

### *Materials*

CAP, purity 98% as determined by high-performance thin-layer chromatography[20], was purchased from E. Merck (Darmstadt, F.R.G.; Lot No. 2394965). DC was synthesized according to Rangoonwala and Seitz[21]. All other chemicals were of reagent grade. The standard stock solutions were made in methanol to give final concentrations of 1 mg/ml, and stored at −20°C. Working standard solutions were made by diluting the stock solution in methanol.

### *Liquid chromatography*

The HPLC-ED system employed consisted of a LC-3A liquid chromatograph (Shimadzu, Kyoto, Japan), a sample injector U-50 (Irica Instruments, Kyoto), a Cosmosil 5Ph separation column (Nakarai Chemicals, Kyoto), 150 × 4.6 mm I.D., and an Irica E-502 amperometric detector with a glassy carbon electrode. As the mobile phase, a degassed solution of 100 m*M* potassium dihydrogenphosphate and 45% acetonitrile (pH 5.0) was used at a flow-rate of 1.0 ml/min. Deionized, doubly glass-distilled water was used for chromatography.

### *Animal experiments and preparation of plasma sample*

Male Wistar rats (180–200 g) were anaesthetized with sodium pentobarbital, and given i.p. CAP (1 mg/kg) in 0.9% saline with 2% ethanol and 10% Tween 80 after i.v. administration of heparinized 0.9% saline via the right thigh vein, as reported previously[6]. The left thigh venous blood was collected at 3- to 7-min intervals into centrifuge tubes placed on ice, and then centrifuged at 13 000 *g* for 2 min. Because CAP in blood is mostly bound to the albumin fraction[6], the venous plasma (200 μl) collected was homogenized in 1.5 volumes of acetone and the precipitate was removed by centrifugation at 13 000 *g* for 2 min. The supernatant was filtered through a 0.45-μm filter (Millipore, Bedford, U.S.A.) and 5–20 μl of the sample were injected for HPLC. CAP in plasma was determined by an internal standard method using DC (20 ng per sample) as standard.

### *Calculation of biological half-life of capsaicin*

The biological half-life, $t_{1/2}$, and rate constant, $k$, of CAP in rats were calculated by fitting with a one-compartment model[22] and least squares regression analysis.

## RESULTS AND DISCUSSION

### *Electrochemistry of capsaicin and dihydrocapsaicin*

Because CAP and DC contain vicinal phenolic and methoxy groups, they readily undergo electrochemical oxidation (Fig. 1). In order to evaluate the applicability of electrochemical detection (ED) to the determination of CAP and DC, information on the electrochemical behaviour of CAP and DC is needed. The necessary information is contained in the voltammograms of CAP and DC. Voltammograms can be obtained with the assistance of HPLC by repetitively injecting a standard solution and stepping the detector potential between injections. Fig. 2 shows voltammograms obtained for CAP and DC under the conditions to be used in their chromatographic separation.

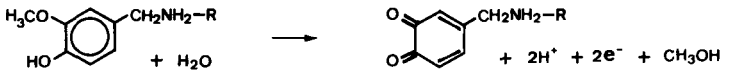


Fig. 1. Oxidation of capsaicin and its analogues. R denotes the $-CO(CH_2)_4CH=CHCH(CH_3)_2$ group of CAP or the $-CO(CH_2)_6CH(CH_3)_2$ group of DC.

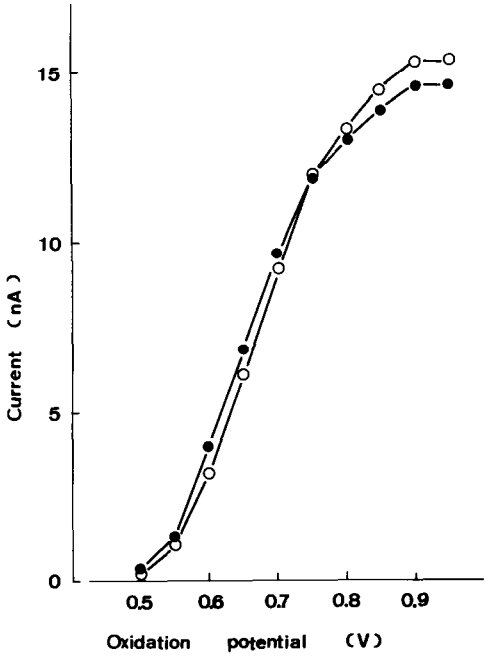


Fig. 2. Voltammograms for capsaicin and dihydrocapsaicin. Conditions: column, Cosmosil 5Ph (5 μm, 150 × 4.6 mm); mobile phase, 100 m*M* potassium dihydrogenphosphate containing 45% acetonitrile; flow-rate, 1.0 ml/min. Standard samples: 5 ng of each compound were injected. ○, Capsaicin; ●, dihydrocapsaicin.

The sensitivity of the detector increased rapidly with the applied potential between +600 and +700 mV, and was almost constant in the range between +850 and +950 mV. A potential of +750 mV *versus* the Ag/AgCl reference electrode was chosen as a compromise between maximum current response and minimum background current.

*Separation of capsaicin and dihydrocapsaicin*

The $k'$ values of CAP and DC were dependent on the acetonitrile concentration in the mobile phase, as shown in Fig. 3. These two compounds can be separated at acetonitrile concentrations lower than 50%. Thus, it is possible to choose an appropriate concentration of acetonitrile for the analysis. We employed a mobile phase containing 45% acetonitrile for routine work.

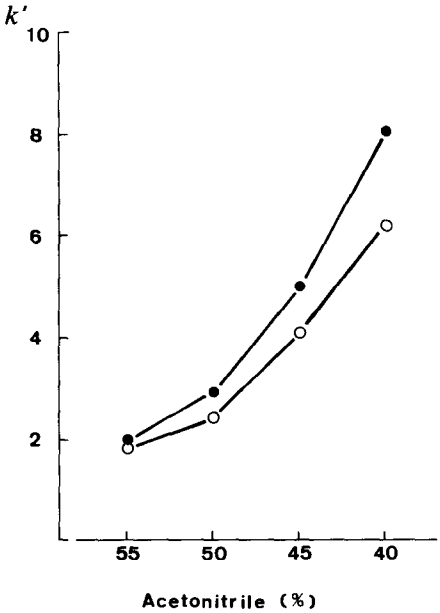


Fig. 3. $k'$ values for capsaicin and dihydrocapsaicin at different acetonitrile–water ratios. The HPLC conditions were as in Fig. 2 under an applied potential of +750 mV, except for the acetonitrile concentration in the mobile phase. ○, Capsaicin; ●, dihydrocapsaicin.

A typical chromatogram of CAP and DC obtained by HPLC–ED is presented in Fig. 4. CAP and DC were effectively baseline-separated and appeared at 8.5 and 10.0 min, respectively, after injection under the conditions described above.

*Linear range and limit of detection*

Calibration graphs for CAP and DC are presented in Fig. 5. Both graphs are linear up to 20 ng per injection, with correlation coefficients of 0.999. The detection limits for CAP and DC were 12 pg per injection at a signal-to-noise ratio of 3:1. The sensitivity for CAP with various detectors reported previously is summarized in Table I. The sensitivity of ED for CAP is much higher than that of UV or fluorescence

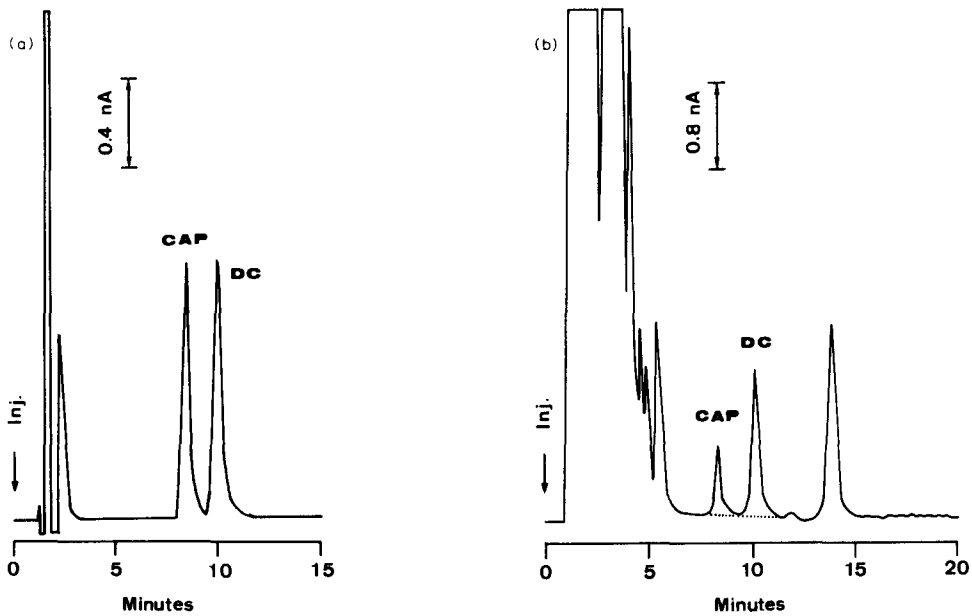


Fig. 4. (a) chromatogram of capsaicin and dihydrocapsaicin. Mobile phase: 100 m*M* potassium dihydrogenphosphate containing 45% acetonitrile (pH 5.0); flow-rate 1.0 ml/min. Applied potential, +750 mV. Sample size: 500 pg of each compound, 5.0 μl. (b) Chromatograms of the extract of rat plasma 16 min after i.p. administration of CAP (1 mg/kg). DC was used as an internal standard. Sample size: 20 μl. ····, Control plasma specimen.

TABLE I

DETECTION LIMITS FOR CAPSAICIN BY HPLC METHODS WITH DIFFERENT DETECTION METHODS

| *Detection* | *Detection limit for CAP* | *Signal-to-noise of detection limit* | *Linearity of detector response, tested up to* | *Ref.* |
|---|---|---|---|---|
| UV (279 nm) | 100 ng | 10:1 | 8 μg | 8 |
| Fluorescence (270/330 nm) | 3 ng | 3:1 | 5 μg | 11 |
| ED (+750 mV) | 12 pg | 3:1 | 20 ng | This study |

detection. So our HPLC-ED system allows most biological samples to be analyzed in very small amounts without a preconcentration step.

*Quantitation of capsaicin in rat plasma*

Fig. 4 shows the elution profile obtained when the plasma of a rat to which CAP had been administered was applied on a Cosmosil 5Ph column and eluted with 100 m*M* potassium dihydrogenphosphate containing 45% acetonitrile at a flow-rate of 1.0 ml/min. Most interfering substances were eluted within the large $V_0$ peak, and effective separation and quantitation of CAP were possible. The recoveries of CAP and DC from the rat plasma were 83.4 ± 4.2% (mean ± S.D., $n = 5$) and 84.1 ± 3.0% ($n = 5$), respectively.

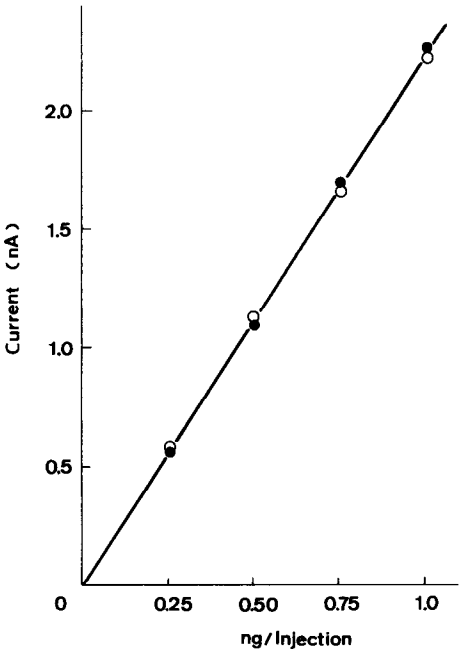


Fig. 5. Calibration graphs for capsaicin and dihydrocapsaicin obtained by HPLC–ED. Samples for each concentration were injected. The HPLC conditions were as in Fig. 4. The results are the mean values for capsaicin (○) and dihydrocapsaicin (●).

*Biological half-life of capsaicin in the rat*

By using this new assay method, the biological half-life of CAP in the rat was investigated. The results obtained showed that the time curve of CAP concentration in plasma can be described by a biexponential equation (Fig. 6). When CAP (1 mg/kg)

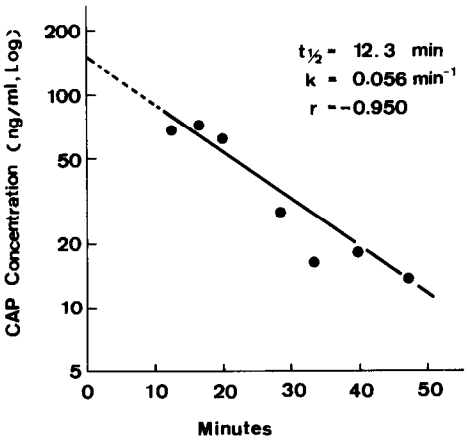


Fig. 6. Logarithm of the plasma concentration of capsaicin as a function of time after i.p. administration. The CAP solution was administered to the rat under light anaesthesia. The thigh venous blood was collected and prepared as described in Experimental, and analyzed by HPLC–ED. $t_{1/2}$ = Biological half-life; $k$ = rate constant; $r$ = regression coefficient.

TABLE II

BIOLOGICAL HALF-LIFE AND RATE CONSTANT OF CAPSAICIN IN THE RAT

The biological half-life and rate constant were calculated by fitting with a one-compartment model as described in Experimental.

| *Rat* | *Biological half-life (min)* | *Rate constant ($min^{-1}$)* |
|---|---|---|
| 1 | 12.3 | 0.056 |
| 2 | 4.26 | 0.163 |
| 3 | 4.33 | 0.160 |
| 4 | 7.36 | 0.094 |
| Mean ± S.D. | 7.06 ± 3.27 | 0.118 ± 0.045 |

was administered to the rat (no. 1), the peak plasma concentration of CAP was 70.4 ng/ml at 16 min, and the concentration at 40 min after the ingestion was 17.7 ng/ml. As shown in Table II, CAP, as well as bromsulphalein and tubocurarine[22], has a very short biological half-life, 7.06 ± 3.27 min ($n$ = 4), and a large rate constant, 0.118 ± 0.045 $min^{-1}$ ($n$ = 4). These pharmacokinetic paramaters of CAP may reflect its hydrophobic properties as reported previously[23,24].

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