Journal of Chromatography, 208 (1981) 41–46
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,534

DETERMINATION OF CAPSAICIN IN TISSUES AND SEPARATION OF CAPSAICIN ANALOGUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received November 19th, 1980)

SUMMARY

Capsaicin, the pungent principle of chili peppers, and six analogues were characterized by reversed-phase high-performance liquid chromatography using isocratic methanol–water mixtures and UV detection. A highly sensitive method for the quantitative determination of capsaicin in animal tissues and blood has been developed. Using a reversed-phase column and acetonitrile–water mixtures for elution, 3 ng of capsaicin could be measured by fluorimetric detection at an excitation wavelength of 270 nm and an emission wavelength of 330 nm. Acetone extraction of tissue resulted in a recovery of approximately 90%. Extraction and determination of capsaicin in rat brain, spinal cord, liver and blood after i.v. administration is given as an example.

INTRODUCTION

Capsaicin, the pungent principle of chili peppers (paprika), is a potent sensory stimulating agent, acting on chemogenic pain receptors[1–4], peripheral and central warmth detectors[5,6] and on pulmonary aortic and carotid baroreceptors[7–9]. Further, it has been found to deplete[10–13] and release [14–16] substance P from chemosensitive neurons. Further actions of capsaicin were reviewed by Virus and Gebhart[17]. Many capsaicin-type substances (amides or esters of homovanillic acid) were found to produce pain[18] and to release substance P from spinal cord slices[19]. For investigations of such structure–activity relationship it is necessary to use very pure compounds. A high-performance liquid chromatographic (HPLC) method[20] was used to characterize capsaicin and six pain-producing analogues for purity control. To elucidate the pharmacological effects of capsaicin, a knowledge of its pharmacokinetic properties is essential. Radioactively labelled capsaicin is not commercially available.

Determination of capsaicin in extracts from *Fructus capsici* by HPLC[21–23] was based on reversed-phase chromatography using water–methanol mixtures for elution with a detection limit of about 100 ng capsaicin. As UV detection does not seem to be sensitive enough for trace determinations of capsaicin in animal tissues, a modified

0021-9673/81/0000–0000/$02.50

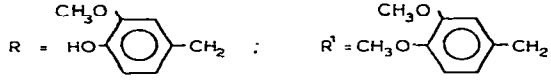
method, based on fluorescence detection, is described here for the determination of capsaicin in rat tissues after systemic administration.

EXPERIMENTAL

Reagents and materials

Capsaicin was obtained from E. Merck (Darmstadt, G.F.R.). Capsaicin analogues (Table I), synthesized according to Hegyes and Földeák[24] or Issekutz *et al.*[25], were a gift from Dr. J. Szolcsányi (Pécs, Hungary). Deionized, double glass-distilled water was used for chromatography. All other reagents were of analytical-reagent grade (E. Merck). All solvents were degassed in an ultrasonic bath prior to use.

TABLE I
STRUCTURES OF INVESTIGATED CAPSAICIN ANALOGUES[18]



No.	*Name*	*Structure*
1	Homovanilloylisobutylamide	R–CONH–CH_2–CH(CH_3)(CH_3)
2	Capsaicin	R–NHCO–$(CH_2)_3$–CH=CH–CH(CH_3)(CH_3)
3	Nonanoylvanillylamide	R–NHCO–$(CH_2)_7$–CH_3
4	Nonenoylhomoveratrylamide	R^1–NHCO–CH=CH–$(CH_2)_5$–CH_3
5	Decanoylhomovanillylamide	R–CH_2–NHCO–$(CH_2)_8$–CH_3
6	Homovanilloyloctyl ester	R–COO–$(CH_2)_7$–CH_3
7	Homovanilloyldodecylamide	R–CONH–$(CH_2)_{11}$–CH_3

Capsaicin solutions

Standards of capsaicin or its analogues (1 mg ml^{-1}) were dissolved in methanol. Dilutions were made in the appropriate eluents. Stock solutions for i.v. injection (10–20 mg ml^{-1}) were prepared with 0.9% sodium chloride solution containing 10% ethanol and 10% Tween. Dilutions for i.v. injections were made with 0.9% sodium chloride solution. The injection volume was 0.5 ml.

Apparatus

The HPLC system consisted of two Altex 110 A pumps with a pulse damper, a Kontron ASI-45 autosampler with a Rheodyne injection valve, an Altex M-420 solvent programmer and a reversed-phase column (Waters μBondapak C_{18}, 300 × 3.9 mm). A Kontron Uvikon LCD 725 UV detector and a Kontron SFM 23 fluorescence detector were connected in series. Recordings of chromatograms and all calculations were performed on a Shimadzu CR1A integrator.

Separation of capsaicin analogues

For separation of capsaicin analogues (Table I), isocratic methanol–water mixtures were used. Detection was performed with a Uvikon LCD 725 UV detector at the absorption maximum of capsaicin of 279 nm[20]. The optimal flow-rate [minimum of $h = f(u)$] of 2.5 ml min^{-1} was used, resulting in a drop in pressure of about 240 bar.

Comparison of water–methanol and acetonitrile–methanol mixtures

Because the viscosity of acetonitrile is lower than that of methanol, a lower drop in pressure with acetonitrile–water than with methanol–water mixtures can be expected[26], various mixtures of these components were compared. Whereas methanol–water mixtures containing 60–80 % of methanol caused a pressure of about 240 bar at 2.5 ml min^{-1} (see above), acetonitrile–water mixtures containing 30–70 % of acetonitrile caused a pressure of only about 160 bar at the same flow-rate. Moreover, for reaching the same k' values of capsaicin lower volumes of acetonitrile were needed (Fig. 1).

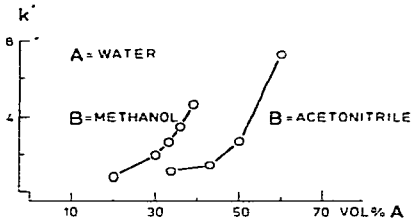


Fig. 1. k' values for capsaicin in methanol–water and acetonitrile–water mixtures. Flow-rate: 2.5 ml min^{-1}. UV detection, 279 nm.

Comparison of UV and fluorescence detection

All separations of capsaicin analogues by HPLC were monitored by UV detection[20–23]. As the sensitivity of UV detection seemed to be too low for the determination of capsaicin in tissues, the sensitivity of fluorescence detection was compared with that of UV detection. For catecholamines and their O-methyl metabolites fluorescence is observed at an excitation wavelength of 250–290 nm and an emission wavelength of 320–340 nm[27]. Under these conditions 5 ng of catecholamines can be detected by HPLC[28]. Owing to the presence of a hydroxymethoxybenzyl group of capsaicin, strong fluorescence could be expected. The optimal fluorescence for capsaicin was found to occur at an excitation wavelength of 270 nm and an emission wavelength of 330 nm. Detection limits and linearity range are shown in Table II.

Extraction of tissues

Sprague Dawley rats of both sexes weighing about 200 g were anaesthetized with 35 mg kg^{-1} of pentobarbitone and 2 mg kg^{-1} of capsaicin was injected into the femoral vein. After 3 or 10 min the animals were killed by bleeding from the carotid artery. Whole brain, about 1 g of liver and whole spinal cord were removed and weighed. Blood and tissues were homogenized in 10 volumes of ice-cold acetone and the homogenate was centrifuged at 3000 *g* for 10 min. The supernatant fluid was evaporated in a Brinkmann sample concentrator and the residue was dissolved in 2.0

TABLE II
COMPARISON OF UV AND FLUORESCENCE DETECTION OF CAPSAICIN STANDARD SOLUTIONS
Elution with 40% acetonitrile in water, flow-rate 2.5 ml min^{-1}.

Detection	*Detection limit* for capsaicin (ng)*	*Linearity of detector response, tested up to*	r^2**
UV (279 nm)	60	20 μg	0.99
Fluorescence (270/330 nm)	3	5 μg	0.99

* At a signal-to-noise ratio of 3:1.
** For nine concentrations.

ml of methanol. After additional protein precipitation by addition of 1.0 ml of 70% perchloric acid, the samples were centrifuged again at 20,000 *g* for 5 min.

RESULTS AND DISCUSSION

Separation of capsaicin analogues

With methanol–water (70:30) all investigated compounds except capsaicin and nonanoyl vanillylamide could be separated (Fig. 2). These two compounds can be separated at methanol concentrations lower than 60% (see *k'* values, Fig. 2).

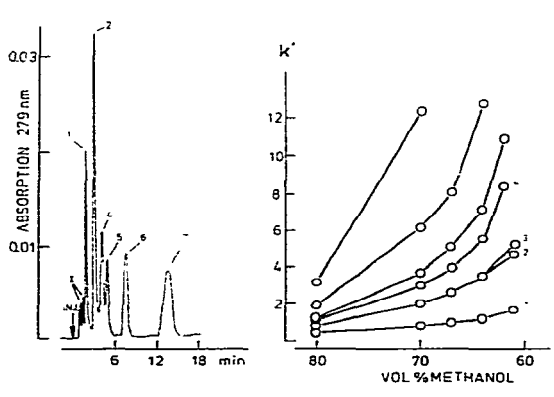


Fig. 2. Left: chromatogram of a mixture of capsaicin and six analogues listed in Table I. Methanol–water (70:30), 2.5 ml min^{-1}. UV detection, 279 nm. 20 μg of each compound, sample size 20 μl. I = impurities of 1. Right: *k'* values with different methanol–water ratios. Other conditions as for the left-hand chromatogram.

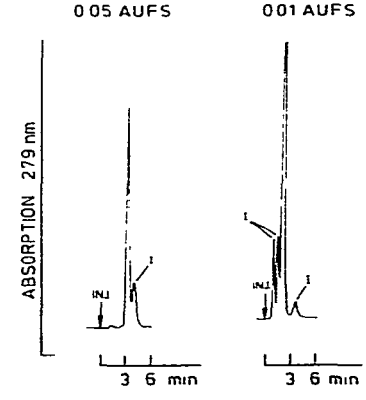


Fig. 3. Chromatograms of 20 μg of capsaicin (left) and 20 μg of homovanilloyl isobutylamide (right). I = impurities. Conditions as in Fig. 2.

The sample of capsaicin used showed a large and a small UV absorbance peak (Fig. 3). The minor peak could be dihydrocapsaicin; an impurity with similar retention behaviour was found in capsaicin from Sigma (St. Louis, MO, U.S.A.) and identified as dihydrocapsaicin[21].

Homovanilloylisobutylamide also showed three unidentified small UV peaks in addition to the main peak (Fig. 3). All other compounds tested gave a single peak.

Quantitative determination of capsaicin in rat tissues

The described extraction method gave recoveries of 88.4 ± 0.61% ($N = 6$) from brain and spinal cord and 86 and 89% from blood and liver. When brain was extracted with methanol instead of acetone the recovery was only 59 ± 2.8% ($N = 3$). The hydrophobic properties of capsaicin allow the use of relatively non-polar elution mixtures. Therefore, most interfering substances are eluted within the large V_0 peak and rapid separation of capsaicin is possible. A simple extraction procedure without further purification of samples prior to chromatography was shown to be

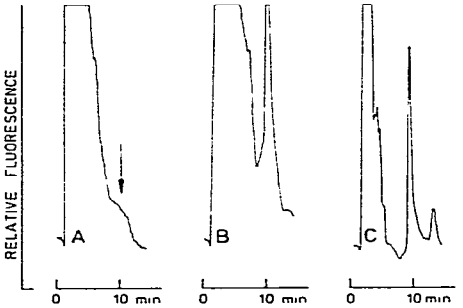


Fig. 4. Chromatograms of rat tissue samples after extraction as described in the text using 40% acetonitrile in water at 2.5 ml min^{-1}. Fluorescence detector, excitation wavelength 270 nm, emission wavelength 330 nm. Sample size, 500 μl. Attenuation for all three chromatograms, "high" position of the fluorimeter; 32 mV full-scale (integrator input). A, Extract of 5 ml of blood of control rat. The arrow indicates the position of elution of capsaicin. B, Extract of 5 ml of blood 3 min after administration of 500 μg of capsaicin i.v. The amount of capsaicin was calculated as 250 ng per 500-μl sample, *i.e.*, 300 ng per gram of blood. C, Extract of 1 g of liver of control rat spiked with capsaicin (100 ng per 500-μl sample).

TABLE III

CAPSAICIN IN RAT BRAIN, SPINAL CORD, LIVER AND BLOOD 3 AND 10 min AFTER i.v. ADMINISTRATION OF 2 mg kg^{-1}

A one-way analysis of variance was performed with all data obtained after 3 and 10 min, followed by Duncan's multiple range test. Values are means ± standard error ($N = 6$).

Time after i.v. administration (min)	*Region*	*Capsaicin (ng g^{-1})*
3	Brain	2763 ± 412*
	Spinal cord	2670 ± 280*
	Liver	1736 ± 355*
	Blood	581 ± 230
10	Brain	364 ± 88**
	Spinal cord	916 ± 192*
	Liver	46 ± 14
	Blood	48 ± 18

* $p < 0.05$ *vs.* blood.
** $p < 0.05$ *vs.* spinal cord.

sufficient. Although the capsaicin peak in blood samples appears on the tail of the broad peak of other fluorescent compounds under the conditions used (Fig. 4), it can be optimally processed by the use of an integrator. An example of the determination of capsaicin in liver is shown in Fig. 4. Similar chromatographic patterns were obtained for brain and spinal cord extracts.

An example of the determination of capsaicin after i.v. administration is given in Table III. Administration of a single dose of 2 mg kg^{-1} of capsaicin i.v. leads to the rapid accumulation of capsaicin in brain, spinal cord and liver compared with blood. After 10 min most of the capsaicin has already been eliminated from blood, liver and brain and only the spinal cord has a significantly higher capsaicin content than blood.

ACKNOWLEDGEMENT

This study was supported by the Austrian Scientific Research Funds, No. 3506.

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