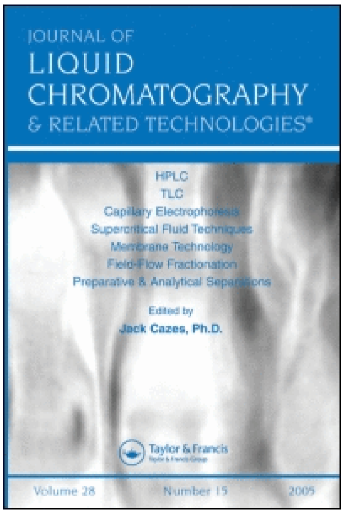

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RAPID ASSAY FOR THE DETERMINATION OF TWO PHOTOACTIVATIBLE KAINIC ACID ANALOGUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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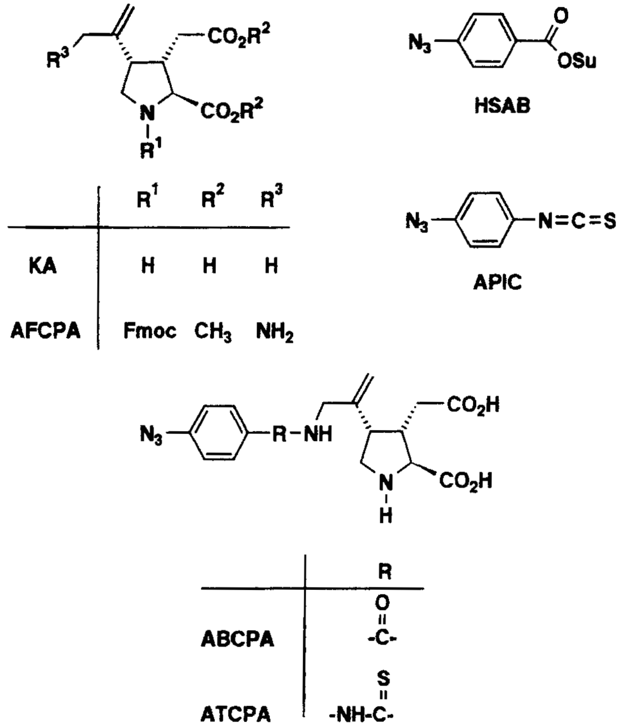
ABSTRACT

An anion-exchange isocratic high-performance liquid chromatographic method for the separation and determination of two kainic acid analogues, namely (2*S*,3*S*,4*S*)-4-[1-(4'-azidobenzoyl)aminomethylethenyl]-2-carboxy-3-pyrrolidineacetic acid and (2*S*,3*S*,4*S*)-4-[1-(4'-azidophenyl)thioureylenemethylethenyl]-2-carboxy-3-pyrrolidineacetic acid is described. These kainic acid analogues were obtained by coupling dimethyl (2*S*,3*S*,4*S*)-4-(1-aminomethylethenyl)-2-carboxy-1-(9-fluorenylmethoxycarbonyl)-3-pyrrolidineacetic acid with N-hydroxysuccinimidyl 4-azidobenzoate and 4-azidophenyl isothiocyanate respectively, followed by saponification. The thus obtained products were purified by reversed phase flash column chromatography and separated on Econosphere -NH_2, using 5% (v/v) acetonitrile in 50 mM acetate buffer pH 5.5, at a flow rate of 1.5 ml/min. The effect of mobile phase pH, and of the acetonitrile concentration on the resolution between kainic acid and its analogues was also studied. Kainic acid was determined by using either low ultraviolet detection (190 nm) or the ninhydrin reaction. Detection of the eluted kainic acid analogues was performed at 215 nm. The detection limit is approximately 1.5 pmol for both kainic acid analogues and the calibration graphs were linear up to 100 nmol (approximately 50-μg).

INTRODUCTION

(-)-α-Kainic acid (KA, Figure 1), a natural product isolated from the marine alga *Digenea simplex*, exhibits powerful neuroexcitatory activity which is attributed to its interaction with specific populations of receptors of the major excitatory neurotransmitters of the mammalian central nervous system, glutamic and aspartic acids [1]. Despite considerable research efforts our knowledge on the molecular properties of these receptors is still far from complete. Since significant progress in the purification and characterization of receptors has been recently achieved using the technique of photoaffinity cross-linking [2], we synthesized KA analogues bearing photolabile moities in different positions of the parent molecule. The most promising of such analogues was found to be the [(2*S*,3*S*,4*S*)-4-[1-(4'-azidobenzoyl)aminomethylethenyl]-2-carboxy-3-pyrrolidineacetic acid, abbreviated here as ABCPA [3]. Using the same methodology (2*S*,3*S*,4*S*)-4-[1-(4'-azidophenyl)thioureylenemethylethenyl]-2-carboxy-3-pyrrolidineacetic acid, abbreviated as ATCPA,



	R^1	R^2	R^3
KA	H	H	H
AFCPA	Fmoc	CH_3	NH_2

	R
ABCPA	$-\overset{O}{\overset{\|}{C}}-$
ATCPA	$-NH-\overset{S}{\overset{\|}{C}}-$

FIGURE 1. Structures of compounds encountered in this report.

was quite recently obtained as briefly described in the experimental part of the present report. Because both analogues showed a significant, but lower than KA itself, specificity for the afore mentioned receptors we decided to study the chromatographical behaviour of these analogues by HPLC against an authentic sample of KA in order to secure that their biological activity was not simply due to the presence of free KA contaminating the synthesized samples.

In this paper we report an isocratic highly sensitive HPLC method in which both KA analogues ABCPA and ATCPA are completely separated from each other as well as from KA.

EXPERIMENTAL

Apparatus and Chemicals

For the determination of KA and its analogues a LDC system with an LDC III pump, a UV-vis detector LDC 1204A set at 215 or 190 nm with 8-μl flow cell and with a 20-μl loop injector was used. The analytical column is a Econosphere $-NH_2$ 5U, 5-μm, 250 x 4.6 mm I.D., stainless steel (Alltech, Deerfield, IL) equipped with an amino guard column, 30 x 4.6 mm I.D. (Brownlee Labs., Santa Clara, CA, U.S.A.).

KA, N-hydroxysuccinimidyl 4-azidobenzoate (HSAB), and 4-azidophenyl isothiocyanate (APIC) were obtained from Sigma Chemical Co. The KA analogues ABCPA and ATCPA were obtained by coupling KA derivative AFCPA (Figure 1) with HSAB and APIC respectively, followed by saponification which resulted in complete deprotection. AFCPA, bearing at nitrogen the commonly used in peptide synthesis 9-fluorenylmethoxycarbonyl (Fmoc) group, was obtained from KA by a multistep reaction sequence [3] involving as a key-step, palladium mediated allylic amination [5]. The thus obtained KA analogues ABCPA and ATCPA were finally purified by reversed phase flash column chromatography [4] on RP-silica octadecyl silane (ODS) which was eluted with distilled water. Full experimental details for the preparation of ATCPA will be published elsewhere. Eluant components were HPLC-grade acetonitrile, obtained from Merck (Darmstadt, Germany) and glass-distilled water. All other chemicals used were of analytical reagent grade.

Chromatographic Conditions

The mobile phase used for the separation of KA analogues ABCPA and ATCPA was 5% (v/v) acetonitrile in 50 mM acetate buffer, pH 5.5. The flow rate was 1.5 ml/min and the pressure 1100 psi. The detection of both KA analogues was performed at 215 nm, whereas for KA at 190 nm. KA was also detected by post-column derivatization using the ninhydrin reaction. The separation was performed at room temperature. The eluent used was degassed by vacuum filtration through a 0.2-µm memrane filter followed by aggitation in an ultrasonic bath.

System Suitability

The column was equilibrated with mobile phase at flow rate of 1.5 ml/min. After a stable baseline was obtained, the standard solutions were injected into the column and the peaks appeared over the increased

TABLE 1

High-Performance Liquid Chromatographic Characteristics for the Determination of Kainic Acid and Photoactivatible Kainic Acid Analogues.

Compound	Retention Time/min (t_R)	Resolution (R_s)
KA	8.5	-
ABCPA	11.0	1.43
ATCPA	13.0	2.57 (1.22)

The resolution between ABCPA and ATCPA is given in parenthesis

retention time. The resolution factors, R_s, were calculated between the chromatographic peak of KA and each individual peak of the KA analogues from the equation $R_s = 2\ (t_2-t_1)/(W_1+W_2)$, where t_2 and t_1 are the retention times of the two peaks and W_1, W_2 are the peak widths at the base of the respective peaks. The resolution factors, R_s, were more than 1.2 indicating complete separation between KA and its analogues as well as between KA analogue ABCPA and ATCPA. This is illustrated in Table 1.

Selectivity

Chromatography of the samples of ABCPA and ATCPA, as obtained by their multistep synthesis from KA, showed no endogeneous interferences at the retention times of KA as well as the respective times of analogues. This indicated that the proposed method could be used in the determination of both KA and its photoactivable analogues without the use of internal standard.

Detection Limit

The detection limits for the KA analogues were estimated as the quantity of these substances producing a signal of the peak height twice the baseline noice. The minimum detectable amount in pmol injected into the column was estimated to be 1.5 pmol (0.75 ng).

Standard Calibration Graphs

Both KA analogues tested were accurately weighed and dissolved in water to give stock solutions of 25 ng/µl each. Standard solutions of 1.0, 5.0, 12.5 and 10.0 ng/µl were prepared by serially diluting the stock solutions. Aliquots of 10-µl were taken for HPLC analysis. The calibration curves were constructed by plotting the peak heights of KA analogues against their concentrations.

RESULTS AND DISCUSSION

The retention times of KA and its analogues ABCPA and ATCPA were reproducible under the chromatographic conditions used with a relative standard deviation of less than 0.5%. The mobile phase used enabled a good column performance for long period of time. On the other

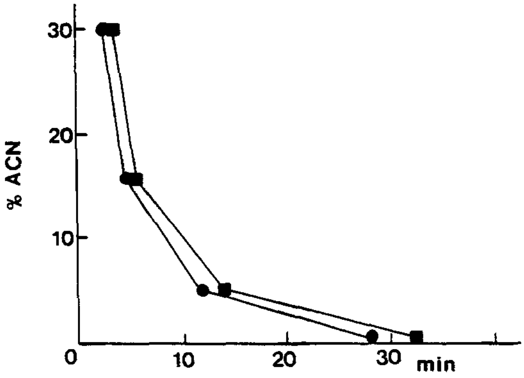


FIGURE 2. Influence of acetonitrile concentration on the retention times of KA analogues ABCPA (●) and ATCPA (■).

hand the KA analogues used were stable at least for several months when kept at 4^0C, under exclusion of light.

The presence of the organic modifier, acetonitrile, in mobile phase gave rise to heigher and more reproducible peaks than the aqueous phase alone thus improving the sensitivity and the accuracy of the described method [6]. As it is shown in Figure 2 the best resolution between the two KA analogues was obtained in concentrations of acetonitrile lower than 5% (v/v), whereas in higher acetonitrile concentrations both KA analogues were coeluted with KA. However, when the peak height were measured in the presence of acetonitrile, it was found that concentrations of more than 5% (v/v) gave heigher

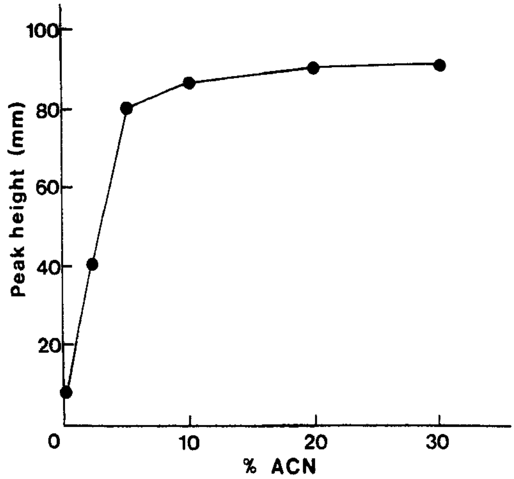


FIGURE 3. Influence of acetonitrile concentration on the peak heights of KA analogue ABCPA (●). The same curve was obtained for KA analogue ATCPA.

peaks (Figure 3). These results indicated that the optimum acetonitrile concentration to use for the separation of KA analogues was 5% (v/v). When pH of the aqueous mobile phase was ranged from 3 to 6 it was found that for pH values 3, 4 and 5 no resolution was obtained, whereas in pH 5.5 and 6 the resolution was complete (Figure 4). However, pH 5.5 was selected for the separation, because it gave the same resolution with pH 6.0 and moreover lower retention times.

Under these conditions, KA as well as its analogues ABCPA *and* ATCPA gave rise to single peaks (Figure 5A, B

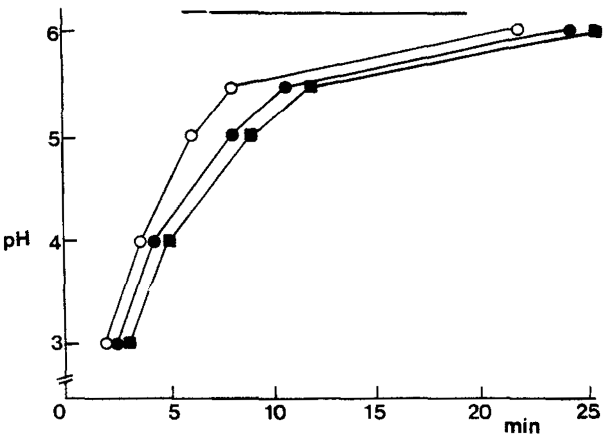


FIGURE 4. Effect of pH of the mobile phase on the retention times of KA (○), ABCPA (●) and ATCPA (■).

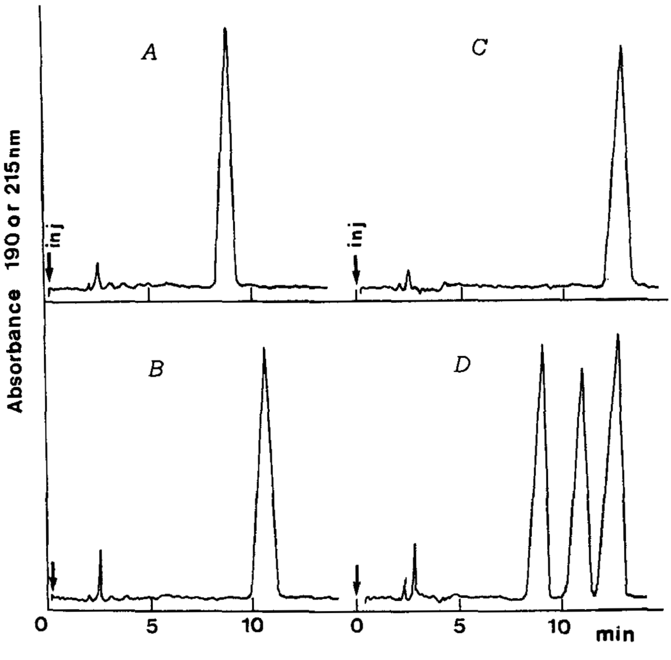


FIGURE 5. Typical HPLC chromatograms of KA (A), ABCPA (B), ATCPA (C) and a mixture of these three compounds (D). KA was detected at 190 nm or with the ninhydrin reaction, whereas the KA analogues were detected at 215 nm.

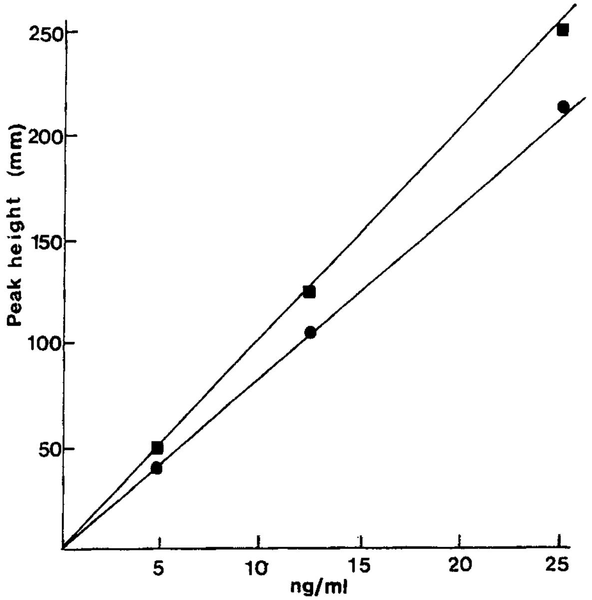


FIGURE 6. Calibration graphs obtained by injecting various amounts of ABCPA (●) and ATCPA (■).

and C). KA was widely separated from the two KA analogues (Figure 5D) in the $-NH_2$ column. Separate chromatographies of KA analogues ABCPA and ATCPA showed the absence of any KA contaminant (Figure 5B and 5C). The front peaks eluted before the compounts of interest were due to the buffer used for column elution (Figure 5).

The sensitivity and the linearity of the method were tested with the use of standard mixtures of various concentrations (Figure 6). The obtained peak heights for the compounds of interest were found to be linear related to the concentration, up to 100 nmol, *i.e.*, when approximately 50-µg of each derivative injected into the column. The precision of the method was determined by six repeated determinations of each derivative. When 15 nmol of each KA derivative were measured, the relative standard deviation was 2.0% for ABCPA and 2.4% for ATCPA, and with 5 nmol the corresponding values were 2.6% and 3.1%, respectively.

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