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Journal of Pharmaceutical and Biomedical Analysis
19 (1999) 967–973

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Short communication

Liquid chromatographic determination of hippuric acid for the evaluation of ethacrynic acid as angiotensin converting enzyme inhibitor

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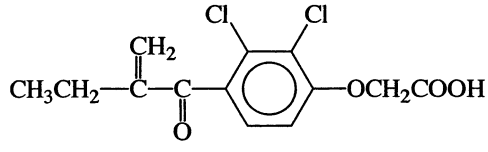
Received 14 November 1997; received in revised form 14 April 1998; accepted 14 April 1998

Abstract

A rapid, simple and interference-free method is described to evaluate the inhibitory effects of organic compounds on the activity of angiotensin converting enzyme irrespective of their acid-base properties. The assay is based on the high performance liquid chromatographic separation of the synthetic substrate hippuryl-L-histidyl-L-leucine, the hydrolysis product hippuric acid and the test compound. Using the new method, the diuretic drug ethacrynic acid was found to act as an inhibitor for the enzyme in a non competitive mode. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Reversed-phase high-performance liquid chromatography; Angiotensin converting enzyme inhibitors; Ethacrynic acid; Hippuric acid

1. Introduction



Ethacrynic Acid

Angiotensin converting enzyme (ACE) is an essential enzyme in regulating electrolyte balance, blood volume and pressure [1]. The enzyme is instrumental in the conversion of the inactive *deca*-peptide, angiotensin I, into the active, potent vasoconstrictor, octa-peptide angiotensin II. Inhibition of this step in the renin-angiotensin system is a very effective strategy in the management of hypertension [2,3]. The structural features of the diuretic drug ethacrynic acid suggest that the drug may have ACE inhibitory properties, as it possesses most of the necessary pharmacophores re-

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PII S0731-7085(98)00122-8

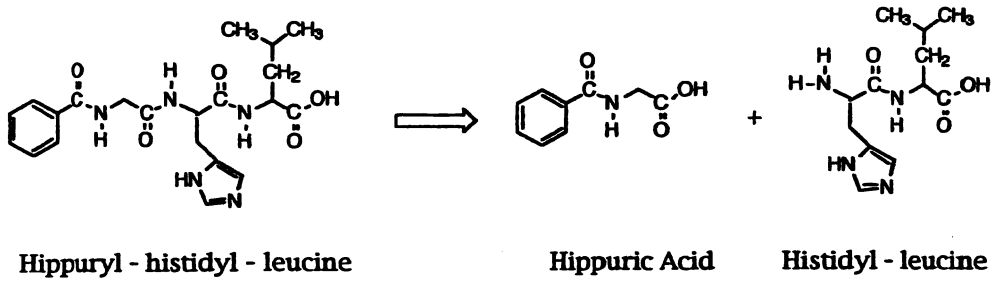

Fig. 1. Hydrolysis of the substrate hippuryl-L-histidyl-L-leucine by angiotensin converting enzyme.

quired to occupy the catalytic active site model described for ACE by Cushman and Ondetti et al. [4,5]. Our initial attempts to test ethacrynic acid for ACE-inhibitory activity using the standard pharmacological screening protocol developed by Cushman and Cheung [6], were unsuccessful. This method involves incubating a mixture of the target inhibitor, the enzyme, and an artificial peptide substrate hippuryl-L-histidyl-L-leucine (HHL) at 37°C. The amount of hippuric acid (HA) produced from the enzymatic hydrolysis of the tripeptide (Fig. 1) was determined by UV absorbance at a wave length of 228 nm following isolation from the assay mixture by ethyl acetate extraction at low pH, evaporation of the organic solvent, and reconstitution of the residue in water. Despite the success of the protocol in assessing ACE inhibitory activity of compounds tested at that time, the isolation of the analyte, hippuric acid, is dependent upon the requirement that all other components must possess basic functional groups in order to be retained in the acidic aqueous phase of the extraction mixture. Accordingly, ethacrynic acid and similar compounds lacking such basic groups are expected to be extracted into the organic ethyl acetate layer and consequently interfere with the UV determination of hippuric acid. Subsequently, several alternative methods were developed to circumvent this problem by using various analytical techniques such as fluorometric, radiochemical, chromogenic and HPLC [7]. Despite the fact that each of these techniques was capable of quantitatively determining ACE activity, each possessed an undesirable element such as complexity of sample preparation, long assay time and high cost of required reagents. The HPLC method developed by Doig and Smiley [7] is a direct injection method for the determination of ACE activity in crude biological samples. Although the method is valuable to monitor ACE activity in plasma and other biological samples and it cleverly reduced the absorbance of the substrate HHL with respect to that of the analyte HA by shifting the wavelength of evaluation to 254 nm, its applicability as a routine protocol for quantitative screening of ACE inhibitors is limited. Partial overlapping of HA and HHL peaks compromises the method selectivity, while the shift of the analytical wave length sacrifices its sensitivity. In addition, the

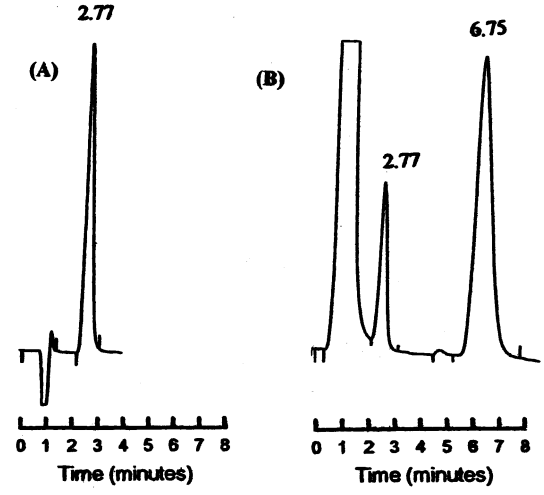

Fig. 2. Representative chromatograms for standard hippuric acid (A), and an actual assay mixture which contained the substrate, ethacrynic acid, and the enzyme incubated as explained in the text.

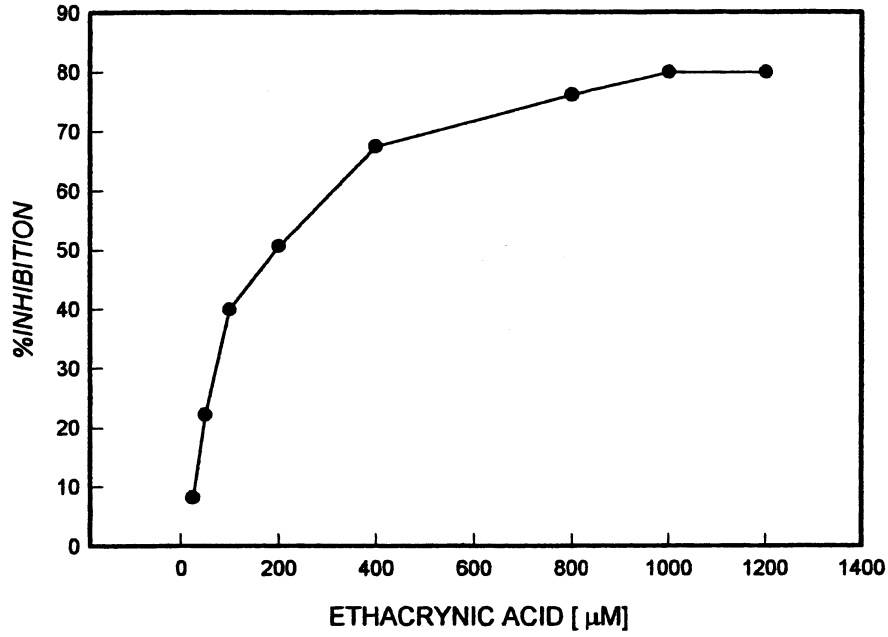

Fig. 3. The inhibitory effects of ethacrynic acid on the activity of the angiotensin converting enzyme. Using the procedure described in Section 2 ethacrynic acid was tested at concentrations of: 0, 25, 50, 100, 200, 400, 800, and 1200 μM, while the substrate concentration was kept constant at 1.0 mM. Percentage inhibition was calculated relative to the enzyme activity of the control runs where no ethacrynic acid was added. Each data point represents the average of four separate runs.

assay requires a costly HISEP SHP (a shielded hydrophobic phase) HPLC column and a centrifugation step for sample preparation, which adds to the cost and analysis time, respectively. Therefore, a rapid, simple, and interference-free liquid chromatographic assay method was developed to analyze the hippuric acid produced by ACE hydrolysis of the substrate hippuryl-L-histidyl-L-leucine (Fig. 1). The method of analysis described here, when combined with the incubation assay of Cushman and Cheung, constitutes a fast and interference-free protocol to evaluate organic compounds for ACE inhibitory activity irrespective of their acid–base properties.

Table 1
Numerical values of the data points in Fig. 3 with standard deviations

Ethacrynic acid concentration (μM)	% Inhibition	SD
25	8.2	1.16
50	22.2	3.00
100	40.0	3.30
200	50.7	2.39
400	67.6	2.11
800	76.2	5.70
1000	80.0	7.50
1200	80.0	7.60

2. Experimental

2.1. Reagents

All reagents including: angiotensin converting enzyme (ACE) from rabbit lung (catalog # A6778), hippuryl-L-histidyl-L-leucine, HPLC grade acetonitrile, ethacrynic acid, hippuric acid, acetic acid and trizma buffer were obtained from Sigma (St. Louis MO).

2.2. Instrumentation

The liquid chromatographic system consisted of a solvent delivery system pump (model 6000A),

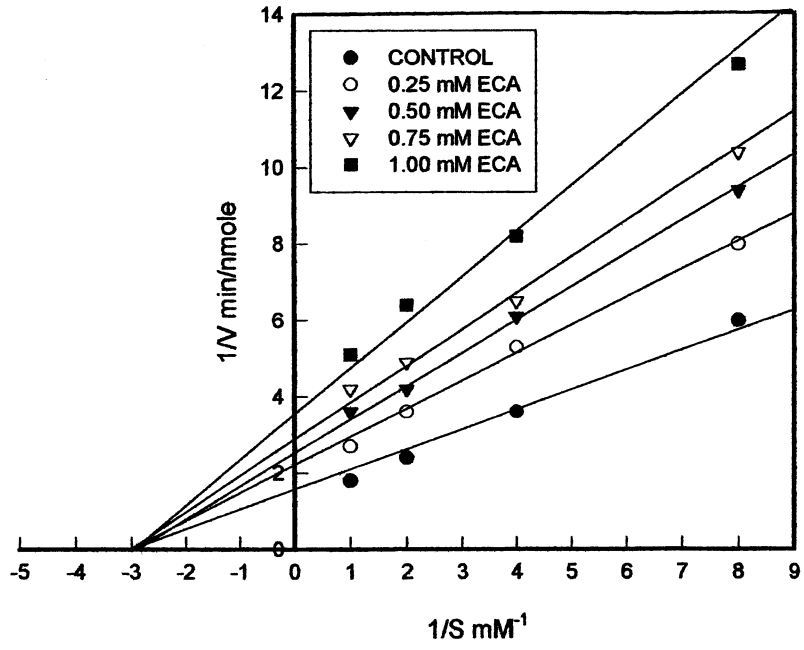

Fig. 4. The Lineweaver–Burke double reciprocal plot for the effects of ethacrynic acid (ECA) concentration on the activity of angiotensin converting enzyme at different substrate levels. Ethacrynic acid was tested at concentrations of: 0.00, 0.25, 0.50, 0.75, and 1.00 mM, each at substrate concentrations of: 125, 250, 500, 1000 μM. The activity of the enzyme in each run was determined as described in Section 2. Each data point represents the average of four separate runs.

(Waters, Milford MA), Rheodyne sample injection valve (model 7012), with 20 μl sample loop (model 7010), (Eglinton Instruments, Amherst, NH), Bondclone 10 μ reverse phase C-18 column (150 × 3.9 mm) protected by a Bondclone 10 μ C-18 guard column (30 × 3.9 mm), (Phenomenx, Torrance CA), Spectroflow model 757 absorbance detector (Kratos Analytical Instruments Ramsey NJ), and an HP integrator model 3390 (Hewlet Packard, Avondale PA).

2.3. Mobile phase

The mobile phase was an isocratic system consisting of 12.5% (v/v) acetonitrile in deionized water. The mobile phase was brought to pH 3.0 by adding 1.0 ml glacial acetic acid per liter of the mixture. The latter was filtered using vacuum through 0.45 μ cellulose filters (MSI, Westboro, MA). The mobile phase was degassed using vacuum for 10 min.

2.4. Chromatographic conditions

The column temperature was ambient (24–26°C) and the detector was set at a wave length of 228 nm. The sensitivity of the detector was set at 1. The integrator was set at peak area mode, attenuation of 2, and threshold of 1, with a chart speed of 0.5 cm min^{-1}. The injection volume was 20 μl and the mobile phase flow rate was 2.0 ml min^{-1}.

2.5. Preparation of standard hippuric acid solutions

A stock solution of hippuric acid of 200 μM (35.84 μg ml^{-1}) in deionized water was prepared and then further diluted with water to give solution concentrations of 25, 50, 75, and 100 μM. The standard solutions were injected in triplicates and the detector response was measured as peak area. The concentration of hippuric acid was extrapolated from a calibration curve of peak area versus standard hippuric acid solutions.

2.6. Validation

The LC method was validated with respect to the following criteria: non-interference of peaks, sensitivity, linearity of response, and precision. Linearity of response was studied by running a standard curve of hippuric acid. Five standard solutions, namely 25, 50, 75, 100 and 200 μM were analyzed to determine linearity. For the determination of sensitivity and limit of detection, dilutions of stock hippuric acid solution were made until no response of the compound peak was observed in the HPLC run. Method precision was carried out by calculating the RSD values of ten separate runs for each of the five hippuric acid standard solutions. Injection precision was carried out by analyzing ten replicates of the 200 μM hippuric acid standard solution. To further validate the method and to determine hippuric acid recovery under the actual incubation assay conditions, the integration values obtained by injecting four hippuric acid concentrations (25, 50, 100, 200 μM in the assay buffer), in the presence and absence of four amounts of the enzyme (1.25, 2.5, 3.75 and 5.0 milliunits), were analyzed.

Table 2
Numerical values of the data points in Fig. 4 with standard deviation

Ethacrynic acid (μM)	1/S mM^{-1}	1 V min^{-1} $nmol^{-1}$	SD
0.00 (Control)	1.0	1.8	0.36
	2.0	2.4	0.14
	4.0	3.6	0.36
	8.0	6.0	0.24
250	1.0	2.7	0.54
	2.0	3.6	0.35
	4.0	5.3	0.24
	8.0	8.0	0.60
500	1.0	3.6	0.30
	2.0	4.2	0.60
	4.0	6.1	0.90
	8.0	9.4	1.26
750	1.0	4.2	0.36
	2.0	4.9	0.54
	4.0	6.5	0.96
	8.0	10.4	0.54
1000	1.0	5.1	1.20
	2.0	6.4	0.30
	4.0	8.2	0.36
	8.0	12.7	0.60

2.7. ACE incubation assay

The enzyme activity was determined using the assay developed by Cushman and Cheung [6] with some modifications. The assay was conducted in Tris buffer (50 mM, pH 8.3) containing 300 mM NaCl. The same buffer was used for all drug, substrate, and enzyme dilutions. A total assay volume of 150 μl was constituted by mixing 50 μl of ethacrynic acid solution with concentration range of 0–3.0 mM, 50 μl of the substrate hippuryl-L-histidyl-L-leucine solution with concentration range of 0–3.0 mM, and 50 μl of enzyme solution containing 1.25 milliunit of enzymatic activity. This resulted in a final concentration range of 0–1.0 mM for both ethacrynic acid and the substrate. All solutions were incubated for 30 min at 37°C in a thermostatically controlled water bath (Fisher Scientific, Pittsburgh PA) prior to mixing, and for an additional 30 min at the same temperature after mixing. The hydrolytic activity of the enzyme was then stopped by adding 150 μl glacial acetic acid to bring the volume to a total of 300 μl which was then directly injected into the sample loop (20 μl loop) to quantify the hippuric acid produced by the enzymatic hydrolysis of the substrate hippuryl-L-histidyl-L-leucine. The enzymatic activity, V, was determined by calculating the amount of hippuric acid produced from the hydrolysis of the substrate expressed as nmoles min^{-1}. The overall method precision (HPLC and incubation assays) was assessed from the standard deviation values for each of the obtained data points.

3. Results and discussion

Fig. 2 shows representative chromatograms obtained after the injection of a standard hippuric acid solution (A), and an actual assay mixture which contained the substrate hippuryl-L-histidyl-L-leucine, ethacrynic acid, and the enzyme (B). The figure shows that the two major peaks in the

Table 3
Recovery of hippuric acid in the presence of variable amounts of ACE[a]

Milliunits of ACE	HA (μM)	Integration without ACE	Integration with ACE	% Recovery
1.25	25	93297	91852	98
	50	182220	186600	102
	100	400930	380880	95
	200	780110	758460	97
2.50	25	91568	98305	107
	50	172820	177710	103
	100	357080	368080	103
	200	790110	748460	95
3.75	25	98448	93564	95
	50	191080	194960	102
	100	370390	387550	104
	200	755140	751340	99
5.00	25	93565	94999	102
	50	190605	181510	95
	100	354310	344550	97
	200	741330	772310	104

[a] Each value is the average of three separate runs.

assay mixture were well and rapidly separated without interference from other assay components or ethacrynic acid. The latter, was found to be retained for a long time on the column and/or the pre-column, and was eluted only during the weekly system wash by 50 and 100% acetonitrile. The separation of hippuric acid and hippuryl-L-histidyl-L-leucine peaks was complete within 7 min with hippuric acid eluting after 2.77 min, and the substrate hippuryl-L-histidyl-L-leucine after 6.75 min. A high signal to noise ratio (S/N) ratio of 26 at hippuric acid limit of detection (LOD) of 5 μM (0.9 μg ml^{-1}) indicates high method sensitivity. The standard curve (constructed by plotting the average of ten separate runs at each hippuric acid concentration level) showed a linear response with a slope of 3848 (standard error of 33), an intercept of -5227 (standard error of 3781), and a correlation coefficient (R^2) of 0.999. The RSD values for each data point of ten separate hippuric acid calibration curves at concentrations of 25, 50, 75, 100, 200 μM were found to be 2.52, 2.87, 2.77, 2.23 and 2.14%, respectively. These low values indicate the analytical method's precision. Likewise, injection precision (studied by injecting ten replicates of 200 μM standard solution) can be concluded from the observed low RSD value of 1.22%.

The method was used to study the inhibitory effects of the diuretic drug ethacrynic acid on ACE activity. The protocol of the incubation assay was essentially the same as developed by Cushman and Chung [6] with minor modifications which allowed more economic use of the enzyme and other reagents. Fig. 3, with the corresponding standard deviation for each data point listed in Table 1, shows that ethacrynic acid indeed acts as an inhibitor for ACE, with an IC_{50} of 200 μM. The figure also shows that 100% inhibition for the enzyme was not reached, even at higher drug concentrations, suggesting a possible noncompetitive mode of inhibition. Fig. 4, with the corresponding standard deviation for each data point listed in Table 2, shows the Lineweaver–Burke double reciprocal plot [8] obtained by studying ACE enzymatic activity under an array of different ethacrynic and substrate concentrations. The study confirmed the noncompetitive type of inhibition, since all lines intersect on the x-axis at $1/K_m$ as shown in Fig. 4. The observed ACE

inhibitory effects of ethacrynic acid, although not a powerful one as reflected by the micromlar range of the IC_{50}, may contribute to the drug's antihypertensive properties which has previously been exclusively attributed to its diuretic effects. It must be indicated that the recovery studies, data summarized in Table 3, indicate that the presence of the enzyme has no effect on the analysis of hippuric acid, even at a four fold higher amount of the enzyme (5.0 milliunits) than the amount used in the current incubation assay (1.25 milliunits). A similar data was obtained in presence of ethacrynic acid at a concentration as high as 1.2 mM. It should also be noted that the periodical removal of adsorbed enzyme and retained ethacrynic acid from the system by weekly column wash with 50 and 100% acetonitrile (300 ml each) helped to maintain good system performance. The same column and pre-column were used throughout the entire study (more than 800 injections) without significant change in the pump back pressure, peak profile or the integration values of standard HA solutions.

4. Conclusions

The newly developed liquid chromatography assay allowed rapid, simple and interference -free analysis of the hippuric acid produced in the ACE incubation assay. The application of the method in studying the inhibitory effects and the mode of inhibition of ethacrynic acid on ACE activity demonstrates its usefulness in testing, when combined with the incubation assay, the inhibitory effects of non-basic and non-peptide organic compounds on the enzyme activity.

Acknowledgements

The authors gratefully acknowledge support of this research from the Glaxo Foundation, Faculty Development Program at the Massachusetts College of Pharmacy and Allied Health Sciences.

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