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Determination of a cysteine protease inhibitor and its ethyl ester in mouse serum and muscle by liquid chromatography–mass spectrometry

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ABSTRACT

A liquid chromatographic–mass spectrometric method is described for the determination of a cysteine protease inhibitor (E64C) and its ethyl ester in mouse serum and muscle samples. The compounds in the sample, after deproteinization and solid-phase extraction, were separated by isocratic reversed-phase high-performance liquid chromatography and detected by on-line mass spectrometry. The use of an aqueous mobile phase containing methanol and 30 m*M* ammonium trichloroacetate provided abundant protonated molecular ions of the compounds in the atmospheric pressure chemical ionization interface of the detection system. The method permitted the quantitative determination of the inhibitors without internal standards in the biological matrices. The detections limits for the compounds, in the selected-ion monitoring mode, were 10–15 pmol on-column, at a signal-to-noise ratio of 5.

INTRODUCTION

A peptide-like compound, (+)-(2*S*,3*S*)-3-[(*S*)-3-methyl-1-(3-methylbutylcarbamoyl)butylcarbamoyl]-2-oxiranecarboxylic acid (E64C) (see Fig. 1) is a potent inhibitor of cysteine proteases, and it has been studied as a possible therapeutic drug for muscular dystrophy [1–4]. However, a positive effect was not always observed when E64C was administered orally to experimental animals [4]. The ethyl ester (EST) (see Fig. 1) of E64C was found to be more effective as an oral drug for the treatment of muscular dystrophy in experimental animals [4].

For pharmacokinetic and medicinal studies, we previously presented the first practical method for monitoring E64C and EST concentrations in mouse muscle and serum by high-performance liquid chromatography (HPLC) with fluorescence detection [5]. We found that EST is metabolized to E64C in the mouse body following oral administration of EST. The method required a rather tedious procedure for the precolumn fluorescence derivatization of E64C and EST with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone; however the sensitive quantification of sub-picomole levels of the compounds was possible.

Recently, HPLC–mass spectrometry (MS) has become a powerful method for quantitative and qualitative analysis of non-volatile biosubstances, such as amino acids, peptides, sugars and nucleotides, because of the great advance of MS interfaces for coupling on-line with HPLC [6–8]. Sakairi and Kambara have introduced a MS interface based on atmospheric pressure chemical ionization (APCI) [9,10]. The APCI-MS interface can handle aqueous mobile phases containing the volatile salts or buffers used for reversed-phase HPLC at a flow-rate of 0.1–2.0 ml/min.

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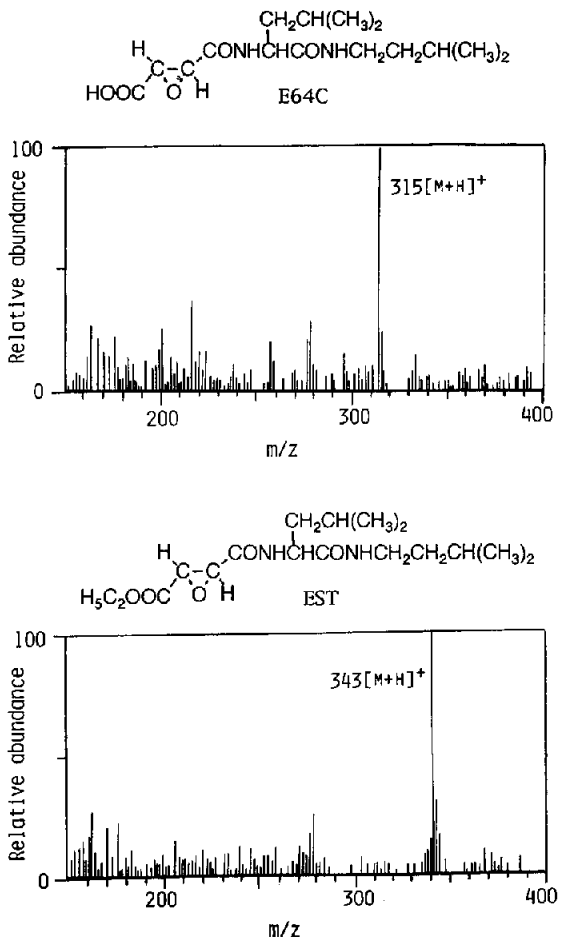

Fig. 1. Molecular structures and mass spectra of E64C and EST. A portion (100 μl) of a standard solution of the compounds (2.0 nmol/ml each) was applied to the HPLC–APCI-MS system [cyclic scan (CS) mode].

This paper demonstrates the applicability of the HPLC–APCI-MS system for the monitoring of the non-volatile compounds, E64C and EST. In this study, the effect on the ionization of E64C and EST in the interface of various volatile electrolytes in the mobile phase was first evaluated, and the quantitative conditions for the determination of the compounds in mouse serum and muscle were optimized by the HPLC–APCI-MS system.

EXPERIMENTAL

Chemicals and solutions

E64C and EST were products of Taisho Pharmaceutical Co. (Tokyo, Japan). The standard solutions (1.0 μmol/ml each) of E64C and EST were prepared in methylcellosolve, and were usable for at least one month when stored at −20°C. The solutions were diluted with water to appropriate concentrations for use within a day. Other chemicals were of the highest purity available. The aqueous solutions of electrolyte salts (Fig. 2) were prepared by mixing equal volumes of the organic acids and bases at equimolar concentrations.

Administration and sample preparation

Inbred mice (C57BL, male, five weeks old, weight 18.5 ± 0.5 g) received a 50 mg/kg oral dose of EST. After the administration, serum and trapezius muscle of the mice were individually collected as described previously [5], and stored at −80°C.

A portion (100 μl) of serum was mixed with 1.0 ml of acetone and 250 μl of water (or a standard solution of E64C and EST). The mixture was centrifuged at 1000 *g* for 15 min for deproteinization. The supernatant was dried *in vacuo*. The residue was dissolved in 1.5 ml of water. The solution was applied to a reversed-phase cartridge (Bond Elut C_{18}; Analytichem Internat., Harbor City, CA, USA; washed with 3 ml each of methanol and water prior to use). After the sample had been loaded, the cartridge was washed with 2 ml of 50 m*M* hydrochloric acid, and then the E64C and EST adsorbed on the cartridge were eluted with 1.0 ml of methanol. After evaporation of the solvent in the eluate *in vacuo*, the residue was dissolved in 200 μl of water–methylcellosolve (1:1, v/v), and subjected to HPLC–APCI-MS.

Sliced muscle (100 mg) was homogenized with 1.0 ml of acetone and 250 μl of water (or a standard solution of E64C and EST). The homogenate was centrifuged at 1000 *g* for 15 min. The supernatant was treated in the same way as the deproteinized supernatant of serum.

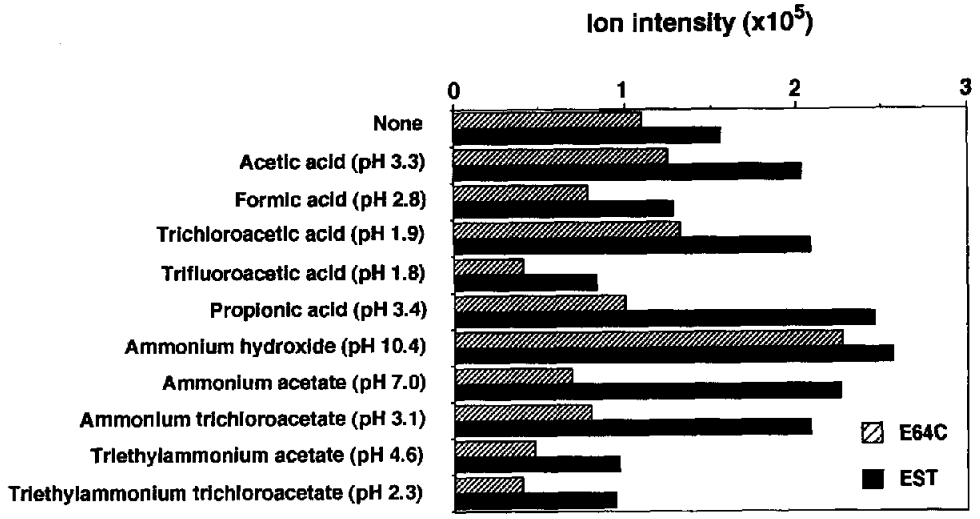

Fig. 2. Effect of 10 m*M* volatile electrolytes in methanol–water (3:7, v/v) eluent on the ionization of E64C and EST. Portions (100 μl) of the standard solutions of the compounds (50 nmol/ml each) were applied separately to the HPLC–APCI-MS system (CS mode) without the HPLC column. The pH values were measured by using 10 m*M* of each aqueous solution.

Apparatus and HPLC–APCI-MS conditions

The HPLC system consisted of a Hitachi L-6200 liquid chromatograph and a Rheodyne 7161 syringe-loading sample injector valve (100-μl loop). A reversed-phase Capcell PAK C_{18} column (250 × 4.6 mm I.D.; particle size, 5 μm; Shiseido, Tokyo, Japan) was used. The column temperature was ambient (25 ± 4°C). The mobile phase was methanol–0.1 *M* ammonium trichloroacetate–water (55:30:15, v/v/v), at a flow-rate of 1.0 ml/min. The column effluent was introduced into a Hitachi M 1000 mass spectrometer equipped with an APCI interface, quadrupole mass analyser and microcomputer data-acquisition system. The instrumentation of the APCI-MS system was previously described in detail [9,10].

For the detection of positive ions by the mass spectrometer, the nebulizer and vaporizer temperatures of the APCI interface were set to 230°C and 390°C, respectively. The applied potential for corona discharge was 3 kV. The drift voltage between the first and second electrodes was 30 ± 10 V. The focus voltage was 130 ± 10 V. The electron multiplier voltage was 1.7 kV. Mass spectra were obtained in the cyclic scan (CS) mode capable of scanning *m/z* 100–400 at 4 s per scan. For quantitative measurement, selected-ion monitoring (SIM) was performed, in which the $[M + H]^+$ ions of E64C and EST were detected alternately for 0.1-s intervals.

RESULTS AND DISCUSSION

Conditions for HPLC–APCI-MS detection

For the HPLC separation of E64C and EST in samples, isocratic elution was performed on a reversed-phase column with an aqueous mobile phase of methanol–water (55:45, v/v) containing 30 m*M* ammonium trichloroacetate; this was necessary for subsequent MS detection. Fig. 1 shows the mass spectra of E64C and EST (200 pmol each on column) obtained under the analytical conditions recommended. The protonated molecular ions, $[M + H]^+$, of both compounds were observed as base peaks in the mass range *m/z* 150–400.

Prior to the measurement of analytes by HPLC–APCI-MS, electronic tuning of the drift (20–40 V) and focus (120–140 V) voltages of the MS interface was always required for the sensitive detection of the $[M + H]^+$ ions of E64C and

EST. The tuning was performed in the SIM mode for their $[M + H]^+$ ions by loading a solution of methanol–water (1:1, v/v) containing 1.0 m*M* E64C and 1.0 m*M* EST into the interface at a flow-rate of 1.0 ml/min without the column. It was also important to control the nebulizer and vaporizer temperatures of the APCI interface [9,10]. The optimized temperatures for the nebulizer and vaporizer were 230°C and 390°C, respectively.

Under these conditions, volatile electrolytes of organic acids, bases or their combination in methanol–water (3:7, v/v) did not drastically affect the $[M + H]^+$ ion abundances of E64C and EST (5.0 nmol each in the interface) (Fig. 2). Unexpectedly, however, an ammonium hydroxide electrolyte promoted most effectively a proton-transfer reaction, especially for E64C, in the interface. In this experiment, the $[M + H]^+$ ions of E64C and EST were always observed as base peaks in their mass spectra.

However, ammonium hydroxide could not be used as the eluent, because the column packing Capcell PAK C_{18} is usable only in the pH range 1–9. When methanol–water (55:45, v/v) containing 10 m*M* ammonium trichloroacetate was used for HPLC–APCI-MS, the calibration curves were linear both for E64C and EST in the concentration range 10–300 pmol on-column (Fig. 3A). However, the calibration curve of E64C was not linear when the methanol–water eluent was used in either the presence or absence of 10 m*M* ammonium acetate (Fig. 3B). We considered that the influence may be caused by pH differences between ammonium trichloroacetate (pH 3.1) and ammonium acetate (pH 7.0), because the dissociation of the acidic compound E64C is suppressed in acidic solution and its hydrophobicity is increased. However, the data obtained with an eluent containing either 10 m*M* trifluoroacetic acid (pH 1.8), or a mixture (pH 3.7) of 10 m*M* ammonium acetate and 10 m*M* acetic acid, did not fit on a straight line for the E64C calibration curve. Therefore, ammonium trichloroacetate was selected as the electrolyte salt for the present HPLC–APCI-MS method.

Fig. 4 indicates the change in the abundance of the $[M + H]^+$ ions of E64C and EST (100 pmol each on-column) when the concentration of ammonium trichloroacetate in the eluent was varied. A concentration of 30 m*M* of the salt was selected for the MS detection. In addition, it was

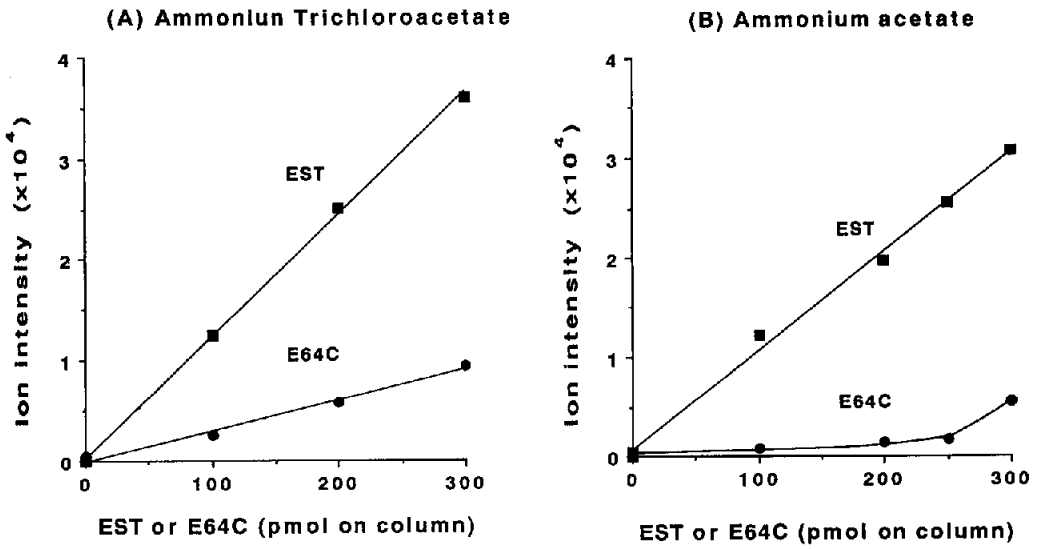

Fig. 3. Calibration curves of E64C and EST obtained by the HPLC–APCI-MS (SIM mode) using methanol–water (55:45, v/v) eluent containing (A) 10 m*M* ammonium trichloroacetate and (B) 10 m*M* ammonium acetate. Two measurements were made for each sample; each plot represents the mean value.

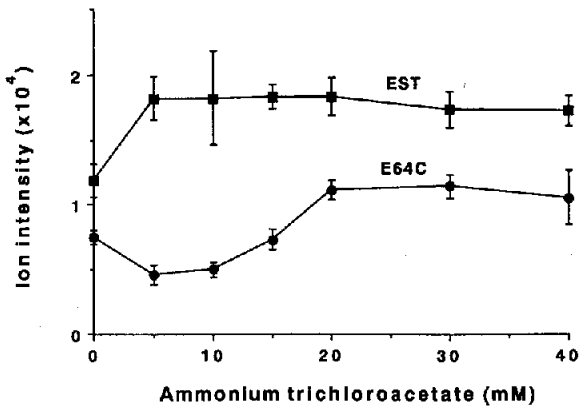

Fig. 4. Effect of ammonium trichloroacetate concentration in methanol–water (55:45, v/v) eluent on the ionization of E64C and EST. A portion (100 μl) of a standard solution of the compounds (1.0 nmol/ml each) was subjected to HPLC–APCI-MS (SIM mode). Two measurements were made for each sample; each point and vertical line represent the mean value and variation level, respectively.

found that other volatile electrolytes, such as acetic acid and ammonium acetate, were also usable when the concentration of the electrolyte in the eluent was increased to 30 m*M*. The increased concentration of either acetic acid or ammonium acetate resulted in the formation of linear calibration curves (0–300 pmol on-column) for E64C and EST. In these cases, however, the $[M + H]^+$ ion abundance of E64C was only 80% or less of that when ammonium trichloroacetate was used. Thus, the ionization effect may vary with the concentration of the electrolyte, depending on the volatility of the electrolyte at the analysing temperature of the interface.

Under the conditions established for the HPLC–APCI-MS analysis, E64C and EST were readily separated on the HPLC column; their retention times were 8.0 and 13.5 min, respectively (Fig. 5). The detection limits for E64C and EST, at a signal-to-noise ratio of 5, were 15 and 10 pmol on-column, respectively. The assay precision was estimated by replicated intra- and inter-assay determinations of E64C and EST (200 pmol each on-column)(Table I). The within-day relative standard deviation (R.S.D.) of the ion intensities of the compounds was smaller than the between-day R.S.D. (7 days), though the mean values were not different statistically. In the present method, intra-assay quantification was performed, based on the standard addition method.

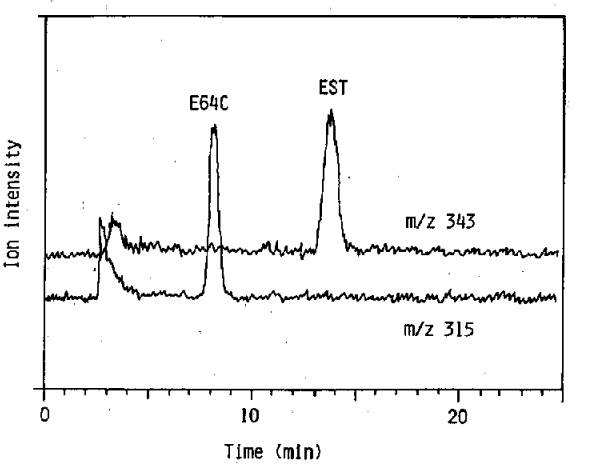

Fig. 5. Mass fragmentograms of E64C and EST in muscle at 1.0 h after oral administration of EST to three mice. Dose: 50 mg/kg. Peaks: E64C: 25 nmol/g; EST: 11 nmol/g.

TABLE I

INTRA- AND INTER-ASSAY REPRODUCIBILITIES IN HPLC–APCI-MS OF E64C AND EST

A portion (100 μl) of a standard mixture of E64C and EST (2.0 nmol/ml each) was applied to the HPLC–APCI-MS (SIM mode). Seven intra-assay measurements were carried out within a day, and one measurement each day was done for one week for the inter-assay.

Number	Ion intensity ($\times 10^5$)			
	Intra-assay		Inter-assay	
	E64C	EST	E64C	EST
1	1.78	8.91	2.14	9.95
2	2.06	10.02	2.27	7.52
3	1.98	9.56	1.94	6.00
4	2.36	10.12	2.07	7.47
5	2.24	9.98	1.55	6.49
6	1.75	8.95	2.35	11.24
7	2.14	9.95	2.58	14.34
Mean	2.04	9.64	2.13	9.00
R.S.D. (%)	10.2	5.0	14.3	30.9

Determination of E64C and EST in mouse specimens

Samples of mouse serum and muscle should be deproteinized and treated with solid-phase extraction, otherwise the HPLC column packing becomes damaged and an unreproducible separation profile is obtained. In addition, the APCI-MS interface was readily smudged with carbon-like materials, especially at the heating nozzle of the nebulizer and the electrode apertures. Acetone was used to deproteinize the samples, because it gave a high recovery (>98%) for E64C and EST [5]. It was not necessary to use a buffer solution to control the pH of the deproteinization process, because E64C and EST in the biological specimens were almost completely recovered from the deproteinization, and the subsequent solid-phase extraction produced the analytes in a small sample size. When the samples were deproteinized with perchloric acid (final concentration 0.2 *M*) in the usual manner, the ester bond of EST was hydrolysed, and then EST was partly converted into E64C in the acidic sample.

Fig. 5 shows chromatograms obtained by the present HPLC–APCI-MS system from the muscle of mice dosed with EST. The E64C and EST peaks in the mass fragmentograms corresponded to their $[M + H]^+$ ions. The calibration curves for E64C and EST in serum and muscle showed a linear relationship between the $[M + H]^+$ ion intensities and amounts of 0.5, 1.0, 2.5 and 5.0 nmol added to the serum and muscle samples. The correlation coefficients of the curves were 0.981 and 0.994 for E64C and EST, respectively; two measurements were carried out for each extracted sample.

The concentration–time graphs for E64C and EST in serum and muscle of mice after oral administration of EST are shown in Fig. 6. E64C, as the active metabolite of EST, was detected in higher concentrations than EST, as demonstrated in a previous paper [5]. The half-life of E64C was 0.4 h in serum and 0.3 h in muscle.

In addition, reconstructed ion chromatograms of the muscle samples, taken 2, 3 and 4 h after the administration of EST, were obtained in order to detect other metabolite(s) of EST by scanning in the mass range of *m/z* 100–400. However, no peaks of metabolites other than E64C could be observed when the chromatograms were compared with those from the drug-free muscle sample.

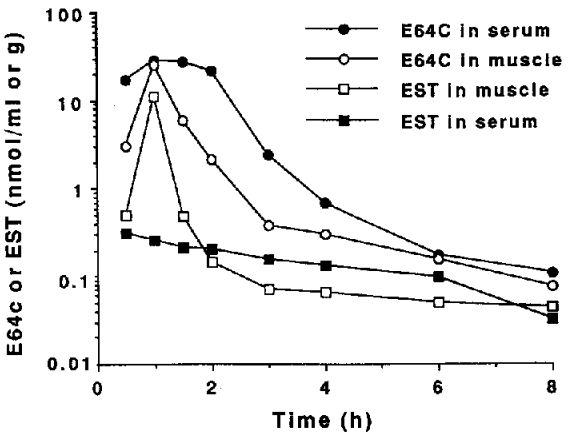

Fig. 6. Concentrations of E64C and EST in serum and muscle of mice ($n = 3$ in each case) after oral administration of EST. Dose: 50 mg/kg. Two measurements were made for each sample; each plot represents the mean value.

CONCLUSION

The APCI-MS interface readily provided the protonated molecular ions of E64C and EST at nanomole amounts in an aqueous methanol mobile phase in the presence or absence of volatile electrolytes. The use of ammonium trichloroacetate in the mobile phase effectively produced straight lines for the calibration graphs of both compounds. The proposed HPLC–APCI-MS method permitted easy quantitative determination of EST and E64C at the picomole level in mammalian tissue and fluid without the use of an internal standard. The sensitivity of the MS detection was *ca.* 10% lower than that of the HPLC fluorescence derivatization method [5]. However, the MS detection possesses a high selectivity without any derivatization and may be convenient for obtaining information about the presence of unknown metabolites in biological specimens.

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