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Short communication

Determination of ethyl-*p*-hydroxybenzoate in sow pancreatic juice by reversed-phase high-performance liquid chromatography

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Abstract

We have developed a high-performance liquid chromatographic–UV–Vis–diode-array detection (HPLC–DAD) method for the determination of ethyl-*p*-hydroxybenzoate, a hydrolytic degradation product of the synthetic protease inhibitor, gabexate-mesilate ethyl-*p*-(6-guanidinohexanoyloxy) benzoate methanesulfonate (GM) (FOY) in sow pancreatic juice. Methyl-*p*-hydroxybenzoate (**I**) was used as the internal standard. The pancreatic juice was deproteinised by acetonitrile and the analytes were chromatographed on a reversed-phase C_{18} LC column using the gradient elution method. The mobile phase consisted of a solution of 0.017 *M* orthophosphoric acid and another solution of acetonitrile–water (80:20, v/v). The wavelength of detection was 237 nm. The limit of quantification of the method was 0.20 μM at a 9:1 signal-to-noise ratio. The overall intra- and inter-day accuracy (relative error, RE) ranged from 14.2 to 8.3% and from 13.3 to 9.8, respectively. The overall intra- and inter-day precision (relative standard deviation, RSD) ranged from 7.6 to 2.62% and from 6.7 to 3.1%, respectively. The method proved to be sensitive, specific, accurate and precise and was successfully used to determine the ethyl-*p*-hydroxybenzoate (**II**) in sow pancreatic juice. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Proteases are widely distributed in the human body and may be involved in several diseases, especially acute pancreatitis. Activated trypsin autodigests the pancreas and acts on kininogen to liberate bradykinin, which activates plasma kallikrein [1]. The liberation of kinin gives rise to systemic shock and pain.

Pharmacological treatment of acute pancreatitis is therefore based on the administration of protease inhibitors, which reduce proteolytic potential within the pancreas [2,3]. Consequently, it is very useful to determine the activities of these substances in body fluids, particularly in the pancreas [4,5]. By determining the activity of protease inhibitors in pancreatic juice, their protective action can be verified. Our research concerning acute pancreatitis has recently focused on the examination of the effect of gabexate-mesilate ethyl-*p*-(6-guanidinohexanoyloxy) benzoate methanesulfonate (GM), a synthetic pro-

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tease inhibitor, in the treatment of pancreatic disease [6]. Due to the greater ease of studying an experimental model, we determined it in sow pancreatic juice. To evaluate the activity of GM, taking into account its rapid inactivation in human blood (half-life 70–80 s), we decided to determine its catabolite ethyl-*p*-hydroxybenzoate in pancreatic juice. Various methods for the determination of the GM catabolism in biological fluids have been proposed.

The use of radioisotopes [7–9] has been widely used since this technique is rapid and sensitive and permits studies on absorption, distribution, extraction and metabolism of GM in several matrices; however, its reduced specificity depending an cross-reactivity and drawbacks due to the use of radioactive compounds led to the proposal of high-performance liquid chromatography (HPLC) as a profitable alternative method [10–12]. Unfortunately, these methods while allowing the determination of ethyl-*p*-hydroxybenzoate in serum or plasma suffer from poor resolution when applied to pancreatic juice, thus impeding the detection of this catabolite in our target organ.

This paper reports the development a high-performance liquid chromatography–UV–Vis–diode-array detection (HPLC–DAD) method for the determination of ethyl-*p*-hydroxybenzoate in sow pancreatic juice. The proposed method may be suitable for monitoring of ethyl-*p*-hydroxybenzoate concentration in biological fluids to assess the optimum dosage of GM.

2. Experimental

2.1. Chemical and reagents

All solvents were of HPLC grade. Acetonitrile and ethanol (95%) were obtained from Carlo Erba (Milan, Italy); orthophosphoric acid was from Merck (Darmstadt, Germany). Water was purified by distillation through a Waters Milli-Q Plus water purification system supplied by Millipore (Waters Chromatography Division, Harrow, UK). Diethylamine (DEA), ethyl-*p*-hydroxybenzoate and methyl-*p*-hydroxybenzoate **I** (internal standard) was purchased from Sigma–Aldrich (Milan, Italy), gabexate-mesilate (FOY) was from Gruppo Lepetit (Milan, Italy).

Individual standard stock solutions of **I** and **II** were prepared in ethanol (95%) at a concentration of 1 mg/ml. Spiking solutions were prepared by diluting of the individual standard stock solutions with ethanol (95%). All the solutions were stored at 4°C and were stable for at least 1 month.

2.2. Pancreatic juice sample collection

We examined eight sows weighing about 130 kg. They were obtained from the Cretone-Rome Farm through the Department of ''Quality and Safety Service on Animal Experimentation'' of the ''Istituto Superiore di Sanità'' in Rome.

They were maintained throughout the study in accordance with the guidelines provided by the Committee on Animal Care of the ''Istituto Superiore di Sanità'' in Rome. This study was performed with the committee's approval, in compliance with the Italian legislation (D.lgs. No. 116, 27 January 1992).

After 16 h of fasting, all sows underwent general anaesthesia. A small sylastic catheter of 8-gauge diameter was inserted into the pancreatic duct. This drainage was linked to the abdominal wall, a suction device was connected and laparatomy closed. The sows received a continuous intravenous infusion of GM at a rate of 1 g/24 h for 24 h.

Pancreatic juice samples were taken 12, 36 and 60 h after the operation. Blank pancreatic juice was obtained from five sows not exposed to GM and equal volumes of each were mixed to obtain pooled blank samples for calibration graphs and recovery studies. After low-speed centrifugation (150 *g* at 4°C for 15 min) the supernatant was collected.

All samples were stored at −20°C until use.

2.3. Extraction procedure

The pancreatic juice samples of treated animals and the fortified samples for calibration graphs and recovery studies were prepared in the same way. Frozen pancreatic juice samples were allowed to reach room temperature and an aliquot of 1.0 ml, 0.20 μM of internal standard and 2 ml of acetonitrile were pipetted into the tubes. The samples were

vortex-mixed for 1 min and then centrifuged at 7000 *g* for 5 min. The supernatant was recovered and dried under a N_2 stream. The residue was redissolved in 200 μl of mobile phase and an aliquot of 50 μl was analysed by HPLC.

2.4. High-performance liquid chromatography

Liquid chromatography was performed by a Beckman Gold System (Beckman Analytical, San Ramon, CA, USA) equipped with an UV–Vis–DAD system, a data acquisition and integration software. The column was a Beckman LiChrospher 100 RP-18 (25×0.5 cm, 5 μm) with a Merck 4-4 HPLC guard column (2×0.3 cm). A loop of 50 μl was used for HPLC injections. Chromatograms were recorded by monitoring the absorbance at 237 nm; peak spectra were obtained with DAD in the range from 190 to 350 nm. Analysis was performed by gradient elution at 25°C with a flow-rate of 1 ml/min.

The gradient composition was: 0.5 min isocratic elution of 90% 0.017 *M* orthophosphoric acid solution adjusted to pH 2.8 (column lifetime is about 6 months) with DEA (solution A) and 10% of acetonitrile–water (80:20, v/v) (solution B); 16 min linear gradient from 10% to 100% solution B; 2.5 min of isocratic elution of 100% solution B; 2 min linear gradient to return to the initial conditions. Mobile phase was freshly prepared for each new sample batch.

2.5. Calibration curve, recovery and reproducibility

Calibration graphs were prepared daily by spiking pooled blank samples with 0.20 μ*M* of internal standard and ethyl-*p*-hydroxybenzoate at concentrations of 0.12, 0.18, 0.24, 0.36, 0.48, 0.72 and 1.20 μ*M*. Calibration graphs were constructed by plotting peak area ratios of ethyl-*p*-hydroxybenzoate to internal standard versus analyte concentration using a linear regression model. These curves were used to determine the analyte concentrations in the samples of treated animals and in the spiked pancreatic juices.

To evaluate the accuracy and precision of the method, five replicates of fortified samples were prepared and analysed on each of 3 days for each concentration (i.e., 0.12, 0.24 and 0.60 μ*M*).

3. Results

Preliminary experiments showed that the adoption of a gradient rather than an isocratic elution gave better chromatographic profiles for the detection of ethyl-*p*-hydroxybenzoate in a complex biological matrix such as pancreatic juice. Representative chromatograms of blank pancreatic juice sample fortified with **I** (0.20 μ*M*) and a blank sample fortified with **I** (0.20 μ*M*) and **II** (0.60 μ*M*) are shown in Fig. 1A and 1B, respectively. The specificity of the method was shown by the absence of endogenous interference around the retention time of the analytes.

The chromatographic separation required 15 min and the resulting peaks were sharp and symmetrical. Identities of peaks **I** and **II** in the pancreatic juice were established on the basis of retention times and of the UV–Vis spectra. They concurred with those of standards (data not shown).

There was a linear relationship between **II** concentration and integrated peak area ratios, as described by the linear regression equation $y=11.431x-0.102$ ($r^2=0.998$), where $y=$**II** peak area/**I** peak area and $x=$**II** concentration. The linearity of the method was evaluated over a concentration range of 0.12–1.20 μ*M*.

The limit of quantification (LOQ), defined as the lowest concentration range of graphs at which an acceptable accuracy and precision are obtained, was 0.20 μ*M* at a 9:1 signal-to-noise ratio. Accuracy and precision data for the HPLC–DAD method were generated each day for 3 days from the analysis of five replicates of fortified blank samples for each concentration (i.e., 0.12, 0.24 and 0.60 μ*M*). The precision of the method was determined by calculating the relative standard deviation (RSD) for the repeated measurements. The accuracy of the method (relative error, RE) was determined by assessing the agreement between the measured and nominal concentrations of the spiked pancreatic juice samples (Tables 1 and 2).

The overall intra- and inter-day accuracy ranged from −14.2 to −8.3% and from −13.3 to −9.8%, respectively. The overall intra- and inter-day preci-

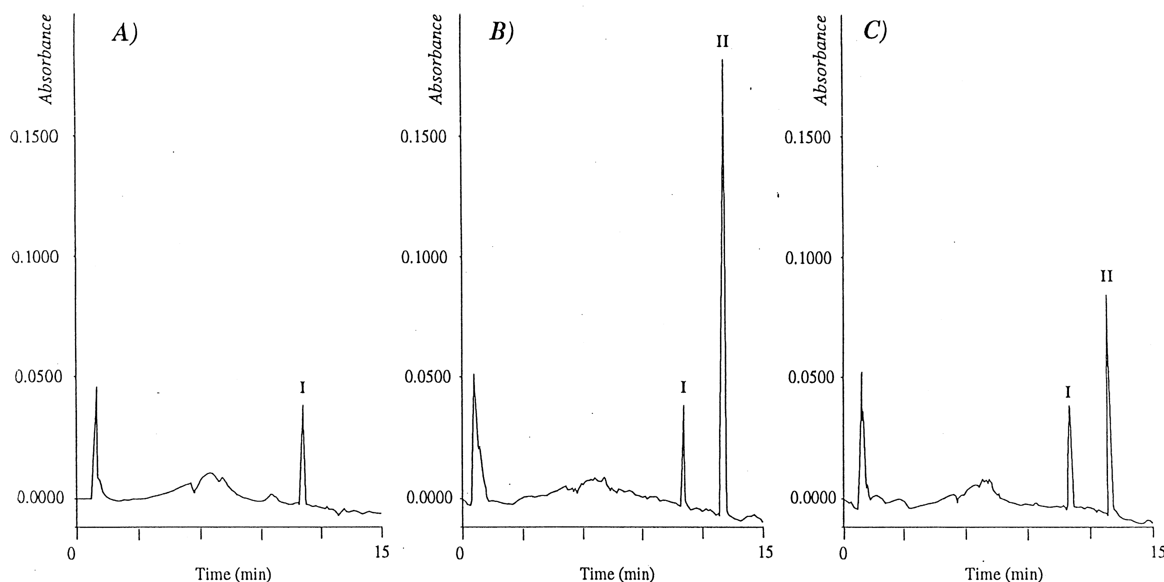

Fig. 1. HPLC chromatograms of: (A) extract of blank pancreatic juice containing methyl-*p*-hydroxybenzoate (**I**) as internal standard (0.20 μ*M*), (B) extract of blank pancreatic juice containing methyl-*p*-hydroxybenzoate (**I**) as internal standard (0.20 μ*M*) and ethyl-*p*-hydroxybenzoate (**II**) (0.60 μ*M*) and (C) extract of pancreatic juice of sow treated with FOY (1 g/24 h) containing methyl-*p*-hydroxybenzoate (**I**) as internal standard (0.20 μ*M*) and ethyl-*p*-hydroxybenzoate (**II**) (0.39 μ*M*).

sion ranged from 7.6 to 2.6% and from 6.7 to 3.1%, respectively. These values are satisfactory considering the complexity of the biological matrix. The HPLC–DAD method was finally used to determine ethyl-*p*-hydroxybenzoate in pancreatic juice of the treated sows. The estimated concentration of the analyte in the treated sows varied from 0.33 to 0.40 μ*M*.

A representative HPLC–DAD chromatogram of a pancreatic juice sample of an animal treated with FOY containing **I** (0.20 μ*M*) and **II** (0.39 μ*M*) is shown in Fig. 1C. The relative retention time of **II** in

Table 1
Intra-day validation statistics for ethyl-*p*-hydroxybenzoate in pig pancreatic juice sample

Day	Parameter	Validation sample level (μ*M*)		
		0.12	0.24	0.60
1	Average concentration found (μ*M*)±SD	0.10±0.01	0.21±0.02	0.55±0.0
	Accuracy (%)	−14.2	−12.5	−8.7
	Precision (%)	6.8	7.6	3.6
	n	5	5	5
2	Average concentration found (μ*M*)±SD	0.10±0.01	0.21±0.01	0.54±0.02
	Accuracy (%)	−13.3	−11.7	−9.8
	Precision (%)	6.7	5.1	2.9
	n	5	5	5
3	Average concentration found (μ*M*)±SD	0.11±0.01	0.22±0.01	0.53±0.01
	Accuracy (%)	−11.7	−8.3	−11.0
	Precision (%)	7.5	4.5	2.6
	n	5	5	5

Table 2
Inter-day validation statistics for ethyl-*p*-hydroxybenzoate in pig pancreatic juice sample

Parameter	Validation sample level (μ*M*)		
	0.12	0.24	0.60
Average concentration found (μ*M*)±SD	0.10±0.01	0.21±0.01	0.54±0.02
Accuracy (%)	−13.3	−10.8	−9.83
Precision (%)	6.7	5.6	3.1
n	15	15	15

all the sow pancreatic juice samples corresponded to that of the calibration standard at a tolerance of ±0.5%.

4. Discussion

Gabexate-mesilate is the first synthetic protease inhibitor used for the treatment of pancreatitis. Due to the presence of ester bonds in the molecule, it can be hydrolysed by plasma esterases. Its biological half-life in human plasma has been estimated to be 70–80 s. To prove the beneficial effect of GM it is important to evaluate its presence and activity in pancreatic juice. Therefore we have determined the concentration of its catabolite, ethyl-*p*-hydroxybenzoate, in pancreatic juice with a new rapid, specific and sensitive HPLC–DAD method.

This method, which was based on previous work [10], can be used to determine ethyl-*p*-hydroxybenzoate in a complex matrix such as pancreatic juice. Until now it has not been feasible to determine ethyl-*p*-hydroxybenzoate due to matrix interferences. The chromatographic conditions described in this manuscript resolve that problem. The assay combines accuracy and good sensitivity: the minimum amount detectable is in agreement with the previously described HPLC methods and has the further advantage of being linear over a wide range. Because of the high sensitivity, the method can be used to measure of the amount of drug in pancreatic juice in humans who have received a therapeutic dose of GM.

Separation and detection of the analyte by HPLC–DAD and the measurement of its concentration, rather than the effect of its enzymatic activity, can be expected to provide more accurate information about the true levels of this catabolite in biological samples. Thus, the presence of ethyl-*p*-hydroxybenzoate in pancreatic juice could suggest that the use of GM is a helpful therapy to the acute pancreatitis.

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