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Gradient high-performance liquid chromatographic analysis of enkephalin peptides, their metabolites, and enzyme inhibitors using combined ultraviolet and electrochemical detection II. Application to ocular permeability studies in vitro

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Abstract

A gradient HPLC method with combined ultraviolet (UV) and electrochemical detection (ED) was used to study the ocular permeability of [D-Ala^2]-methionine enkephalinamide (MEA) in vitro. Coulometric ED was selective for MEA and its tyrosine-containing metabolites with quantitation limits between 20 and 60 n*M* (1.0–3.0 pmol per 50-μl injection), whereas UV detection at 205 nm allowed the determination of several aromatic metabolites and enzyme inhibitors with quantitation limits between 40 and 500 n*M* (2.0–25.0 pmol). This method was capable of detecting permeability of MEA and metabolite formation in the cornea and conjunctiva in vitro. Furthermore, effects of aminopeptidase inhibitor bestatin and enkephalinase inhibitor SCH 39370 on permeation and metabolism of MEA could be determined. © 1998 Elsevier Science B.V.

Keywords: Enkephalin peptides; Enzyme inhibitors; Methionine enkephalinamide

1. Introduction

Peptide chemistry offers many potential uses in the treatment of ocular diseases [1,2]. However, peptides are usually poorly absorbed due to their low permeability in ocular membranes and their enzymatic degradation. For example, two analgesic pentapeptides, leucine enkephalin (LEK) and methionine enkephalin (MEK) (Tyr–Gly–Gly–Phe–X; X is Leu or Met), were extensively metabolized and less than 1% of the dose was absorbed into ocular tissues in rabbits [2]. The absorption of an enkephalin analog, [D-Ala^2]methionine enkephalinamide (MEA; Tyr–D-Ala–Gly–Phe–Met–NH_2), was in the same range, although it was more resistant to enzymatic degradation than the native peptides [2].

Ocular absorption of peptides might be improved by reducing their metabolism during absorption with coadministration of enzyme inhibitors. In ocular tissues, LEK and MEK are mainly hydrolysed by aminopeptidases with a smaller contribution of dipeptidyl peptidase and dipeptidyl carboxypeptidase that is most probably enkephalinase [3]. However, an aminopeptidase inhibitor bestatin did not improve the ocular absorption of MEK in rabbits [2]. A better

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approach might be to use a more stable enkephalin analog with a combination of enzyme inhibitors. MEA is 10–20 times more stable to hydrolysis than LEK and MEK in ocular tissues [3]. MEA is principally degraded to Tyr–D-Ala–Gly by dipeptidyl carboxypeptidase, probably enkephalinase, and to a lesser extent to Tyr by aminopeptidases, so the inhibitors of these peptidases may improve the ocular absorption of MEA.

Ocular absorption of enkephalins in vivo has been determined in rabbits using radiolabelled peptides [2]. In ocular permeability studies in vitro, a more convenient HPLC–UV method has been used to measure conjunctival permeability of MEA and its metabolites [4]. However, in order to study the corneal permeability of MEA in vitro, a more selective and sensitive method is needed, since the cornea is less permeable to macromolecules than conjunctiva [5]. Liquid chromatography with electrochemical detection (LC–ED) is a selective and sensitive method for enkephalins and their tyrosine-containing metabolites without any derivatization. Several isocratic LC–ED methods have been developed for these compounds [6–9], but these methods are not able to detect the parent peptide and all the possible metabolites in the same run. In the present study, we applied gradient HPLC method with combined UV and ED detection [10] to study the effects of bestatin (aminopeptidase inhibitor) and SCH 39370 (enkephalinase inhibitor) on the corneal and conjunctival permeability of MEA in vitro.

2. Experimental

2.1. Chemicals

Amino acids and peptides were purchased from Sigma (St. Louis, MO, USA) with following exceptions. Tyr–D-Ala was obtained from Haartman Institute (University of Helsinki, Helsinki, Finland), and Tyr–D-Ala–Gly–Phe from Bachem (Bubendorf, Switzerland). Enzyme inhibitors bestatin and thiorphan were from Sigma. Captopril was kindly donated by Bristol-Myers Squibb (Princeton, NJ, USA), and SCH 39370 (*N*-[*N*-[1-(*S*)-carboxyl-3-phenylpropyl]-(*S*)-phenylalanyl]-(*S*)-isoserine) [11] by Schering-Plough (Kenilworth, NJ, USA). Acetonitrile (HPLC Super grade) was purchased from Rathburn (Walkerburn, UK). Phosphoric acid 85% (H_3PO_4) and sodium dihydrogen phosphate dihydrate (NaH_2PO_4) (both of analytical grade) were from Merck (Darmstadt, Germany). Other chemicals were of analytical grade. Water was distilled and further purified with Millipore-Q UF Plus apparatus (Molsheim, France). Molecular masses of analytes (Da): Tyr, 181; Tyr–D-Ala–Gly, 309; MEA, 587; bestatin, 308; SCH 39370, 414.

2.2. Equipment

HPLC was performed with two Beckman 114 M pumps connected to a high-pressure mixing chamber, and a Beckman 420 system controller (Berkeley, CA, USA). Stainless steel reservoir filters were replaced with polyethylene filters (Upchurch, Oak Harbor, WA, USA) to avoid the leaching of electroactive metal ions, and standard PTFE tubes of the pumps were replaced with FEP tubes (DuPont, Wilmington, DE, USA) with a much lower oxygen permeability. A Scientific Systems LP-21 LO-Pulse pulse damper (State College, PA, USA) was connected after the mixing chamber. Samples were introduced with a Spectra-Physics 8775 autosampler (San Jose, CA, USA) with a 50-μl sample loop. The dwell volume between the mixing chamber and column inlet was 1.5 ml.

UV detection was performed with Waters 486 UV detector (Milford, MA, USA). An ESA Coulochem 5100A electrochemical detector (Bedford, MA, USA) was used with an ESA 5014 flow cell. The flow cell contains two flow-through working electrodes (E1 and E2) made of porous graphite (E1, 4–5 cm^2; E2, 1 cm^2; total cell volume, 4 μl). The ESA 5014 flow cell was connected after the UV detector and induced 20 bar back-pressure at 1 ml/min. In addition, an ESA Model 5020 guard cell was connected between the pulse damper and the autosampler to pre-electrolyze the mobile phase. Both cells were equipped with ESA graphite in-line filters (0.2 μm). The ESA cells contain a solid-state palladium reference electrode and the operating potentials are typically 0.3 V lower than with a Ag–AgCl reference electrode. Signals from the detectors were collected at 1 Hz by Borwin Chromatography Software (Le Fontanil, France).

2.3. Chromatographic conditions

Reversed-phase HPLC was performed on a Kromasil C_8 (5 μm, 100 Å, 150×4.6 mm) column (Eka Nobel, Bohus, Sweden). Solution A was 35 m*M* phosphate buffer (pH 2.1), and solution B was 59 m*M* phosphate buffer (pH 2.1)–acetonitrile (60:40, v/v). Solution A was prepared by mixing 35 m*M* H_3PO_4 solution and 35 m*M* NaH_2PO_4 solution until pH 2.1 was obtained. The phosphate buffer in solution B was prepared in a similar manner using 59 m*M* solutions, and finally acetonitrile (ACN) was added (final concentration of phosphate buffer in solution B is 35 m*M*). Solutions A and B were filtered (Durapore 0.22 μm, Millipore, Bedford, MA, USA), and degassed by sparging with helium. Separations were performed at ambient temperature at the flow-rate of 1.0 ml min^{-1}.

In the gradient elution of MEA, its metabolites, and enzyme inhibitors bestatin and SCH 39370, the ACN content of the mobile phase was increased linearly from 4 to 5% during the first 9 min, then to 15% during 5 min, and finally to 30% during 21 min. After the 35-min run, the ACN content was returned to 4% during 3 min, and the column was re-equilibrated for 17 min before the next injection.

The UV detector was set to 205 nm (filter time 0.5 s). The first (screen) electrode of the ESA 5014 flow cell was set to 0.25 V, the second (analytical) electrode to 0.60 V (response time 2 s, gain 1×1), and the ESA 5020 guard cell to 0.65 V. The electrodes were equilibrated for 24 h before the analysis. The optimization of separation and detection was described in an earlier paper [10].

2.4. Calibration, accuracy and precision

Stock solutions of the amino acids, peptides, and enzyme inhibitors (0.5–1.0 m*M*) were prepared in water, divided into aliquots, and stored frozen (−20°C) until used. SCH 39370 did not dissolve until the pH of the solution was adjusted to 6.6 with 0.1 *M* NaOH.

Calibration standards and quality control (QC) samples were prepared in glutathione bicarbonated Ringer's (GBR) solution [12] which is commonly used as a medium in ocular permeability studies in vitro. GBR solution contains inorganic salts, glucose (5 m*M*), and oxidized glutathione (0.15 m*M*), and the pH of the solution is 8.0–8.1. All the solutions were prepared in bulk, divided into aliquots, and stored frozen (−20°C) until analysed.

Calibration standard mixtures containing Tyr, Tyr–D-Ala–Gly, and MEA were prepared with concentration ratios of 1, 2, 4, 10, 100, 333, and 1000, with lowest concentration of 30 n*M* of Tyr, 60 n*M* of Tyr–D-Ala–Gly, and 20 n*M* of MEA, respectively. When bestatin and SCH 39370 were used in the permeability studies, these inhibitors were added to the calibration standards with concentration ratios of 1, 10, 33, and 100, with lowest concentration of 500 n*M* of bestatin and 250 n*M* of SCH 39370, respectively. Calibration curves were calculated by linear weighted $(1/X)$ regression on the peak heights versus concentrations. The only exception was the ED calibration curve of MEA that was slightly exponential (see Section 3.1.1.). Peak heights were used, since they were more reproducible than peak areas at low concentrations.

QC samples containing MEA, seven of its possible metabolites, and enzyme inhibitors bestatin and SCH 39370, were prepared with concentration ratios of 1, 5, 10, and 100 (see Fig. 1 for the concentrations of the compounds in the highest QC sample). The stock solutions of Tyr–D-Ala–Gly and MEA used for QC samples were prepared from different peptide batches than the stock solutions used for calibration standards. In the accuracy and precision tests of Tyr, Tyr–D-Ala–Gly, and MEA, QC samples were compared daily to calibration curves. In addition, QC samples were used as calibration standards in the determination of other metabolites of MEA.

2.5. In vitro permeability studies

In vitro permeability of MEA in cornea and in conjunctiva of New Zealand rabbits was studied in perfusion chambers as previously described [5]. The exposed surface areas of the cornea and conjunctiva were 1.17 and 0.28 cm^2, respectively. The test solution was either 1.0 m*M* MEA in GBR solution or 1.0 m*M* MEA with 0.25 m*M* bestatin and 0.25 m*M* SCH 39370 in GBR solution at pH 7.65. GBR solution (6.5 ml) was added to the receptor side and immediately thereafter, an equal volume of the test solution was added to the donor side. Samples of 0.3 ml were collected from the receptor side at 10, 30, 60, 90, 120, 180, and 240 min and replaced with

equal volume of blank GBR solution. The samples were stored frozen (−20°C) until analysed by HPLC without any extraction procedure. In addition, the test solutions and samples taken from the donor side at 4 h were analysed both undiluted using only UV detection (to avoid the contamination of the analytical electrode of ED) and as 2% dilutions in GBR solution using combined UV and ED detection.

2.6. LC–MS analysis

LC–MS analysis of thiorphan, captopril, and their reaction products in GBR solution was performed with a Rheos 4000 pump (Flux Instruments, Danderyd, Sweden), a Rheodyne 7725 injector (Cotati, CA, USA) with a 20-μl sample loop, and a LCQ electrospray-ionization (ESI) mass spectrometer (Finnigan MAT, San Jose, CA, USA) operating in the positive mode. The ESI needle voltage was 5.6 kV, and the sheat gas (nitrogen) value was 95. A Purospher RP-18e (5 μm) sorbent in a LiChroCART (125×3 mm) cartridge (Merck, Darmstadt, Germany) was used for the separation. Solution A was water–acetonitrile (95:5, v/v) containing 60 m*M* formic acid, and solution B was water–acetonitrile (30:70, v/v) containing 60 m*M* formic acid. Separations were performed at ambient temperature at a flow-rate of 0.5 ml min^{-1}. With this method, the retention order of the compounds was the same as with the method described in Section 2.3. However, in isocratic runs, the ACN content of the mobile phase was reduced by 5% compared with the method in Section 2.3. to obtain adequate retention for all the compounds.

LC–MS identification of Tyr–Gly and Tyr–Gly–Phe–Met–NH_2 (see Section 3.2.1.) was performed with the same apparatus, except that the column was Purospher RP-18e (5 μm, 125×2mm) (Merck, Darmstadt, Germany) and the flow-rate was 0.2 ml min^{-1}.

3. Results

3.1. Method validation

3.1.1. Calibration curves

MEA, seven of its possible metabolites, and enzyme inhibitors bestatin and SCH 39370, were separated with gradient elution (Fig. 1). The calibration curves of Tyr and Tyr–D-Ala–Gly were linear ($r>0.998$) and reproducible in both UV and ED detection (Table 1). The calibration curve of MEA was also linear ($r>0.999$) in UV detection, but slightly exponential in ED detection. MEA differs from Tyr and Tyr–D-Ala–Gly, since it contains two electroactive residues, Tyr and Met–NH_2. The sulfur atom of Met–NH_2 residue is oxidized at a higher potential than the phenolic group of Tyr residues [10]. To improve the selectivity of ED detection, the operating potential was chosen from that part of the voltammogram where the sulfur atom of Met–NH_2 residue is only partially oxidized [10]. The contribution of the sulfur atom to the exponential response was supported by the fact that another sulfur-containing peptide Tyr–D-Ala–Gly–Phe–Met (without the amide group in the carboxy terminal) also gave slightly exponential response (data not shown), whereas Tyr, Tyr–D-Ala–Gly, and Tyr–D-Ala–Gly–Phe gave linear responses. The exponential response of MEA did not cause any problems, since it was easily handled with the data system. The ED response of MEA was not as stable as that of Tyr and Tyr–D-Ala–Gly, but changes were slow and therefore good accuracy and precision was obtained (see Section 3.1.2). For all Tyr-containing compounds, the lower limit of dynamic range of ED detection was slightly lower than that of UV detection (Table 1). On the other hand, unlike UV detection, the linear dynamic range (exponential for MEA) of ED detection did not extend to the highest calibration standards used.

Bestatin and SCH 39370 were determined only by UV detection due to their low electroactivity [10]. The calibration curves (weighted $1/X$) of bestatin (range 0.5–50.0 μ*M*) and SCH 39370 (range 0.25–25.0 μ*M*) were linear ($r>0.999$).

3.1.2. Accuracy, precision, quantitation limits and detection limits

Accuracy and precision were studied by analysing QC samples (Table 2). ED detection offered reliable results (R.S.D.<10%) at the level of the lowest calibration standards, and therefore these are the practical limits of quantitation (LOQ). The LOQ of MEA (20 n*M*) corresponds to 0.002% permeability of the peptide, when the initial concentration of MEA in the donor cell is 1.0 m*M*. The detection

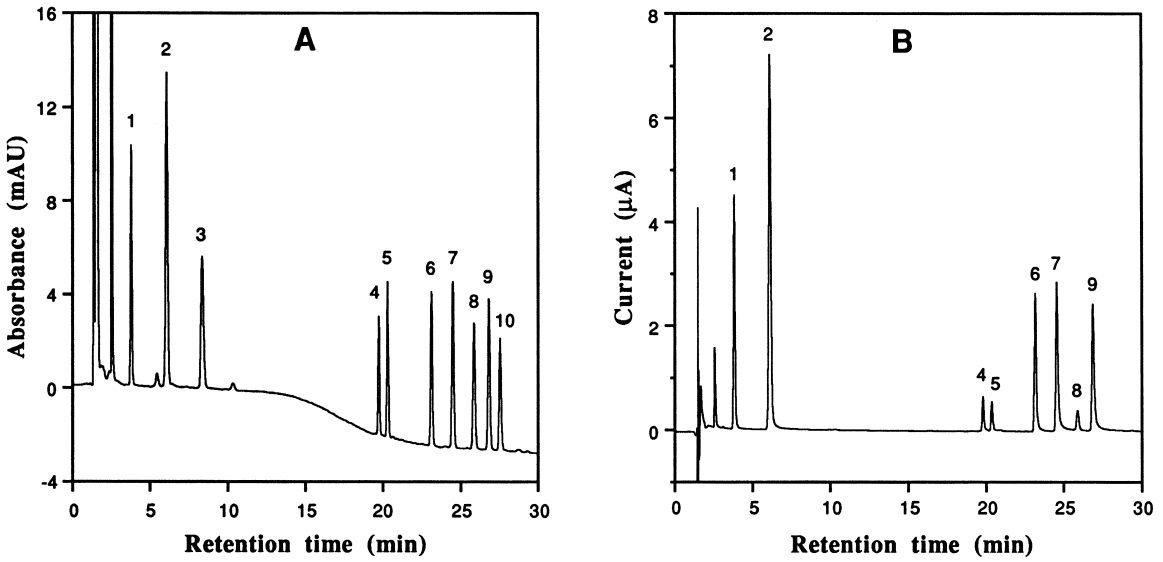

Fig. 1. Gradient elution of a mixture of MEA, its possible metabolites, and enzyme inhibitors in GBR solution (the highest quality control sample). (A) UV detection at 205 nm, (B) ED detection at 0.60 V. Peaks: (1) Tyr (3 μ*M*); (2) Tyr–D-Ala–Gly (6 μ*M*); (3) Phe (40 μ*M*); (4) Phe–Met (8 μ*M*); (5) D-Ala–Gly–Phe–Met–NH_2 (6 μ*M*); (6) Tyr–D-Ala–Gly–Phe (2 μ*M*); (7) MEA (2 μ*M*); (8) SCH 39370 (12 μ*M*); (9) Tyr–D-Ala–Gly–Phe–Met (2 μ*M*); (10) bestatin (20 μ*M*). (Additional retention time: Tyr–D-Ala 10.1 min). Column, Kromasil C_8 (150×4.6 mm). Mobile phase, solution A is 35 m*M* phosphate buffer (pH 2.1) and solution B is 59 m*M* phosphate buffer (pH 2.1)–acetonitrile (60:40, v/v). Acetonitrile concentration was increased from 4 to 5% in 9 min, then to 15% in 5 min and finally to 30% in 21 min. Flow-rate, 1.0 ml min^{-1}. Guard cell potential, 0.65 V.

limits of Tyr-containing compounds in ED detection (S/N 3) were 8–12 n*M* (0.4–0.6 pmol per 50-μl injection).

For the two highest QC samples, the average concentrations of the compounds obtained by UV and ED detection were close to each other (Table 2). UV detection was more precise than ED detection for the highest QC sample. In UV detection, the LOQs of Tyr-containing compounds and enzyme inhibitors bestatin and SCH 39370 were the same as the lower limits of calibration range, and were between 40 n*M* of MEA and 500 n*M* of bestatin (see Table 1 and Section 3.1.1). At this level, the R.S.D. of peak heights during a 3-day batch was less than 10% (n=3–6). The detection limits of UV detection (S/N 3) ranged from 20 n*M* (1.0 pmol) of MEA to 240 n*M* (12 pmol) of bestatin.

3.1.3. Chemical stability of peptides and enzyme inhibitors in GBR solution

The chemical stability of MEA in GBR solution was studied using the same conditions as in permeability studies in vitro, but without any ocular membrane. Two concentrations of MEA, 0.325 and 3.25 μ*M*, were selected from the range of typical permeability samples. The concentration of MEA

Table 1
Calibration curves (n=6)

Compound	UV detection		ED detection	
	Range (μ*M*)	Equation[a]	Range (μ*M*)	Equation[a]
Tyr	0.12–30.0	$y=(324\pm13)x+(2\pm29)$	0.03–10.0	$y=(1494\pm46)x-(20\pm24)$
Tyr–D-Ala–Gly	0.12–60.0	$y=(217\pm5)x+(11\pm13)$	0.06–6.0	$y=(1134\pm91)x-(7\pm54)$
MEA	0.04–20.0	$y=(320\pm5)x-(9\pm16)$	0.02–6.7	$y=(940\pm176)x\ \exp(1.08\pm0.05)$

[a]Linear weighted ($1/X$) regression on peak heights versus concentrations, except the ED calibration curve of MEA that is exponential.

Table 2
Accuracy and precision of the analytical method (n=6)

Compound	Nominal concentration (μM)	UV detection			ED detection		
		Mean found (%)	Within-day R.S.D. (%)	Between-day R.S.D. (%)	Mean found (%)	Within-day R.S.D. (%)	Between-day R.S.D. (%)
Tyr	0.03	—[a]	—[a]	—[a]	113.9	2.0	4.4
	0.30	105.1	1.2	1.9	100.9	1.2	3.6
	3.00	102.3	1.0	2.6	98.8	0.7	3.1
Tyr–D-Ala–Gly	0.06	—[a]	—[a]	—[a]	92.9	2.9	7.0
	0.60	97.4	0.8	3.4	95.7	0.5	2.6
	6.00	97.5	0.5	1.7	96.9	0.9	5.3
MEA	0.02	—[a]	—[a]	—[a]	100.3	2.7	6.9
	0.20	109.4	1.1	5.5	106.2	0.8	5.0
	2.00	105.7	0.5	2.7	107.7	1.4	6.6

[a]Not determined, since the concentration is below the lowest calibration standard of UV detection.

remained the same during the 4 h test, and after subsequent 24 h storage at room temperature in the autosampler carousel. QC samples containing MEA, seven possible metabolites of MEA, and enzyme inhibitors bestatin and SCH 39370, were also stable for 24 h in the autosampler and for 3 months in a freezer (−20°C).

On the other hand, well-known thiol-containing enzyme inhibitors thiorphan and captopril were not stable in GBR solution. When GBR solution containing 50 μM of thiorphan was stored at room temperature, the concentration of thiorphan decreased to 47 and 6% of the initial level in 1 and 5 h, respectively. In the gradient elution with phosphate buffer–ACN, two large peaks with equal peak heights appeared in the UV chromatogram at 24.3 and 24.8 min, about 9 min before thiorphan (33.7 min). The ED response of these compounds was very weak compared to thiorphan, which indicated that they did not contain a free thiol group. LC–MS analysis revealed that these compounds were mixed disulfides of thiorphan and glutathione with a $[M+H]^+$ ion at m/z 558.9 (calculated 559.2). These compounds were apparently formed in a thiol–disulfide exchange reaction between thiorphan and oxidized glutathione (present initially at 120 μM in the GBR solution of this experiment). Two diastereomers were formed, since thiorphan is a racemate.

An isocratic analysis with phosphate buffer–ACN (65:35, v/v) revealed that thiorphan disulfide was also formed in GBR solution. At these conditions, the mixed disulfides were eluted as a single peak at 2.0 min, remaining thiorphan at 4.9 min, and two new peaks with equal peak areas at 11.2 and 13.7 min. LC–MS analysis indicated that the latter compounds were diastereomers of thiorphan disulfide with a $[M+H]^+$ ion at m/z 504.9 (calculated 505.1). After 10 h of reaction, only 4% of initial level of thiorphan was left. In addition, the total UV peak area (205 nm) of the mixed disulfides was 7.7 times larger than the combined peak area of the enantiomers of thiorphan disulfide. This might be explained by the high initial concentration of oxidized glutathione compared to thiorphan which favors mixed disulfide formation over thiorphan disulfide formation.

The reaction pattern of 50 μM captopril in GBR solution (containing 140 μM of oxidized glutathione in this experiment) was similar to thiorphan, except that captopril is an optically pure enantiomer. Therefore, in the gradient elution with phosphate buffer–ACN, the mixed disulfide of captopril and glutathione was eluted as a single peak at 18.9 min, remaining captopril at 22.6 min, and the captopril disulfide at 32.7 min. The compounds were identified by LC–MS. The $[M+H]^+$ ions of mixed disulfide and captopril disulfide were at m/z 523.1 (calculated 523.2) and 432.9 (calculated 433.1), respectively.

After 2 h of reaction, only 30% of captopril was left, and the UV area of mixed disulfide was 20 times larger than the area of captopril disulfide.

Thiorphan and captopril could not be used in the permeability studies due to their poor chemical stability in GBR solution. Instead, chemically stable inhibitors bestatin and SCH 39370 were chosen for the permeability experiments.

3.2. In vitro permeability studies

3.2.1. Corneal permeability of MEA

Corneal permeability of MEA was low without enzyme inhibitors. The final concentration of MEA in the receptor cell after the 4 h experiment was 63±56 n*M* (n=5) which is 0.0063±0.0056% of the initial concentration of MEA in the donor cell (1.0 m*M*). As seen in Fig. 2, MEA was extensively metabolized. The major metabolites were Tyr and Tyr–D-Ala–Gly which suggests that aminopeptidases and enkephalinase participated in the hydrolysis of MEA. However, control experiments with a cornea and plain GBR solution revealed that about half of the Tyr was released from cornea.

ED detection was more selective than UV detection for MEA and its Tyr-containing metabolites (Fig. 2). In the ED chromatogram in Fig. 2B, only two impurities (peaks X and Y) are seen. Peak X was a matrix component, since it was also found in control experiments with a cornea and plain GBR solution. Peak Y was an unknown impurity with a 0.3-min longer retention time than Tyr–D-Ala–Gly–Phe standard. In addition, the lack of UV response in Fig. 2A indicated that peak Y was not a Tyr–containing peptide. In ED detection, the concentration of MEA in the receptor cell reached the LOQ at 30–180 min after the beginning of the test, except in one experiment where the concentration of MEA remained below the LOQ even at 4 h. With UV detection, MEA could be quantitated 30–60 min later than with ED detection, except in two experiments where the concentration remained below the LOQ. When the levels of MEA, Tyr and Tyr–D-Ala–Gly were above the LOQ of UV detection, the concentrations of each compound obtained by ED and UV detection were usually within 10% of each other indicating that the peaks were pure.

When bestatin (aminopeptidase inhibitor) and SCH

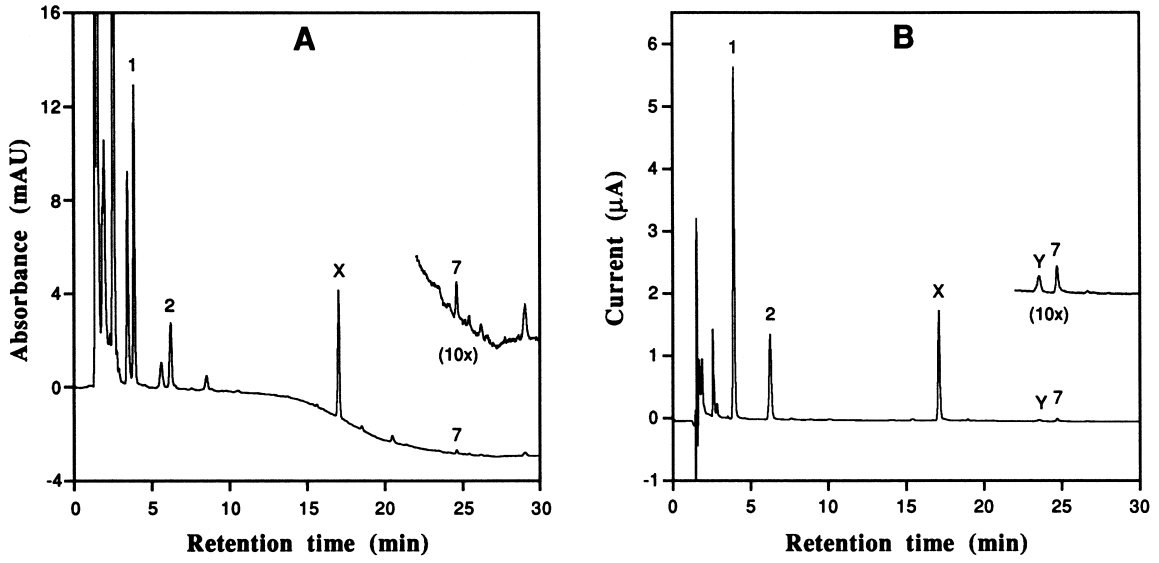

Fig. 2. Analysis of MEA and its metabolites in the receptor cell at the end (4 h) of in vitro corneal permeability experiment without inhibitors. (A) UV detection at 205 nm, (B) ED detection at 0.60 V. Peaks: (1) Tyr (3.95 μ*M*); (2) Tyr–D-Ala–Gly (1.27 μ*M*); (7) MEA (0.048 μ*M* corresponding to 0.0048% permeability); X, matrix component; Y, unknown impurity. Initial donor cell concentration: MEA 1.0 m*M*. Conditions as in Fig. 1.

39370 (enkephalinase inhibitor) were added to the donor cell at the concentration of 0.25 m*M*, the corneal permeability of MEA increased by 11 times with a reduction in the metabolism. The concentration of MEA in the receptor cell at 4 h was 710±430 n*M* (0.071±0.043%) (n=6). As seen in Fig. 3, the high selectivity of ED detection was useful in the determination of the lowered concentrations of Tyr and Tyr–D-Ala–Gly. The enzyme inhibitors were detected with UV detection in the receptor cell in four of the six experiments, but their concentration at 4 h was either below or only slightly above the LOQs (see Fig. 3A). However, the permeability of the inhibitors (% permeated at 4 h) in these experiments seemed to be slightly higher than that of MEA.

In the donor cell, Tyr and Tyr–D-Ala–Gly were the major Tyr-containing metabolites of MEA after the 4 h test and their concentration was markedly reduced with the inhibitors (data not shown). Unexpectedly, Tyr–Gly was found in all the experiments in the donor cell after the 4 h test, but not in the test solutions before the incubation or in the receptor cell. Tyr–Gly (t_R 4.0 min) was partially resolved from Tyr (t_R 3.8 min) with the original HPLC method. However, when the ACN content of the mobile phase was kept at 2%, Tyr and Tyr–Gly were completely separated with t_R of 5.8 and 6.4 min, respectively. Tyr–Gly was identified in the donor cell samples by LC–MS with a $[M+H]^+$ ion at 239.0 (calculated 239.1). LC–MS was used to clarify the origin of Tyr–Gly. A small peak (about 1% of MEA) was observed at m/z 516.2 in the test solution before the experiment. This exactly corresponds to the calculated $[M+H]^+$ ion of Tyr–Gly–Phe–Met–NH_2, a possible synthesis impurity. Probably, Tyr–Gly–Phe–Met–NH_2 was enzymatically hydrolysed at Gly–Phe bond during the test with a release of Tyr–Gly that may further be degraded to Tyr.

3.2.2. Conjunctival permeability of MEA

Conjunctiva was much more permeable to MEA than cornea. Without enzyme inhibitors, the concentration of MEA in the receptor cell at 4 h was

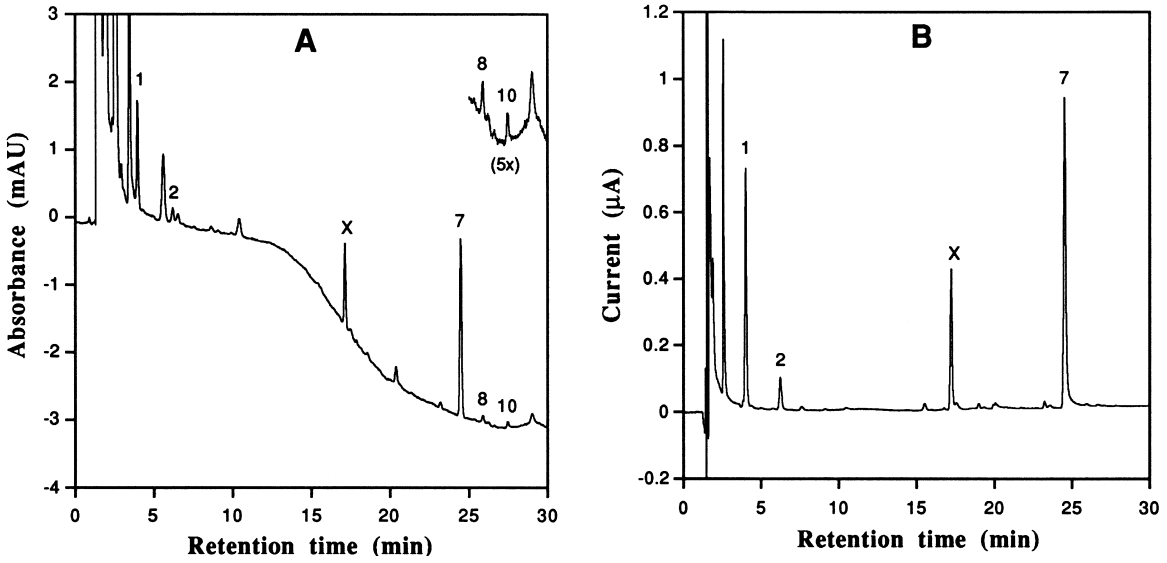

Fig. 3. Analysis of MEA, its metabolites, and enzyme inhibitors in the receptor cell at the end (4 h) of in vitro corneal permeability experiment with inhibitors. (A) UV detection at 205 nm, (B) ED detection at 0.60 V. Peaks: (1) Tyr (0.448 μ*M*); (2) Tyr–D-Ala–Gly (0.082 μ*M*); (7) MEA (0.754 μ*M* corresponding to 0.0754% permeability); (8) SCH 39370 (ca. 0.25 μ*M*, 0.10% permeability); (10) bestatin (ca. 0.35 μ*M*, 0.14% permeability); X, matrix component. Initial donor cell concentrations: MEA 1.0 m*M*, bestatin 0.25 m*M*, SCH 39370 0.25 m*M*. Conditions as in Fig. 1.

1.78±0.55 μ*M* (0.178±0.055%) (*n*=6), about 30 times higher than with cornea even though the exposed surface area of conjunctiva was 4 times smaller compared to cornea. In the conjunctiva, Tyr and Tyr–D-Ala–Gly were formed, but the metabolism was not as extensive as in cornea (data not shown). The combination of bestatin and SCH 39370 increased the conjunctival permeability of MEA 5 times by reducing the metabolism. The final concentration of MEA in the receptor cell was 9.94±3.74 μ*M* (0.994±0.374%) (*n*=5). A typical chromatogram is shown in Fig. 4. At the end of the experiment, the concentration of MEA was typically determined by UV detection, since it was above the dynamic range of ED detection. However, Tyr and Tyr–D-Ala–Gly were selectively determined by ED detection. Bestatin and SCH 39370 were determined by UV detection and their permeability (% permeated at 4 h) were 1.4 and 1.3 times higher than that of MEA, respectively. This is expected, since the molecular weights of bestatin (308) and SCH 39370 (414) are smaller than that of MEA (587) and the inhibitors are not susceptible to metabolism.

4. Discussion

MEA was chemically stable in glutathione bicarbonated Ringer's (GBR) solution (pH 8) in the time scale of the permeability experiment and analysis (24 h). The GBR solution is often used in the ocular permeability studies in vitro to preserve tissue integrity of ocular membranes [4,12]. On the other hand, thiol-containing enzyme inhibitors thiorphan and captopril reacted rapidly with oxidized glutathione, a component in GBR solution, forming mixed disulfides. In the experiments with the initial concentration of 50 μ*M* of thiorphan or captopril in GBR solution, the half-lives of the compounds were less than 2 h at room temperature. Therefore, thiorphan and captopril could not be used in the permeability studies due to their poor chemical stability in GBR solution. Instead, chemically stable inhibitors bestatin and SCH 39370 were chosen for the permeability experiments.

In the present study, a recently developed gradient HPLC method with combined UV and ED detection [10] was used to determine the ocular permeability

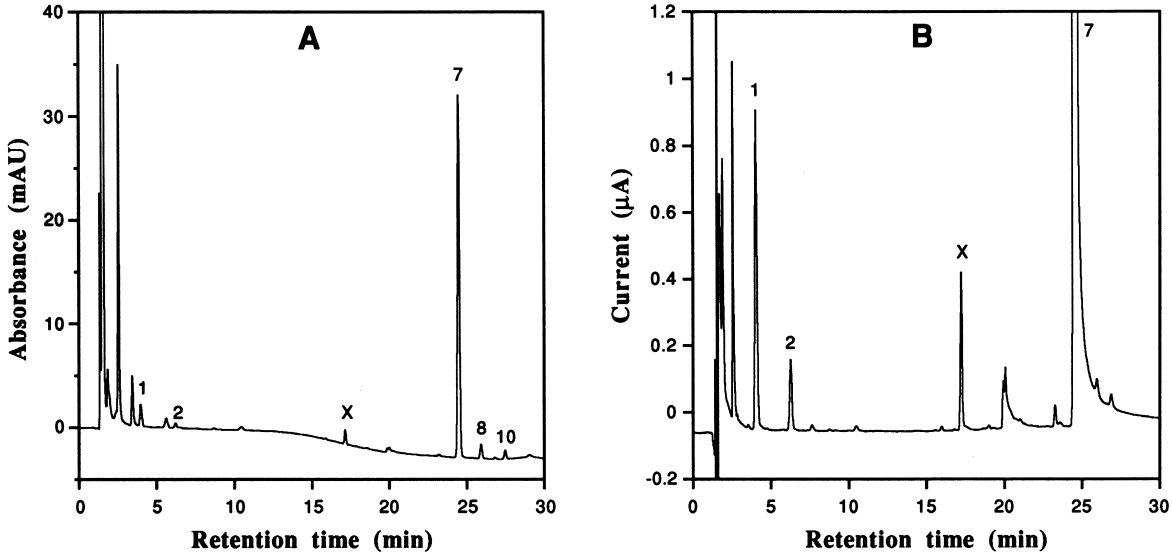

Fig. 4. Analysis of MEA, its metabolites, and enzyme inhibitors in the receptor cell at the end (4 h) of in vitro conjunctival permeability experiment with inhibitors. (A) UV detection at 205 nm, (B) ED detection at 0.60 V. Peaks: (1) Tyr (0.599 μ*M*); (2) Tyr–D-Ala–Gly (0.177 μ*M*); (7) MEA (10.9 μ*M* corresponding to 1.09% permeability); (8) SCH 39370 (3.54 μ*M*, 1.42% permeability); (10) bestatin (4.03 μ*M*, 1.61% permeability); X, matrix component. Initial donor cell concentrations: MEA 1.0 m*M*, bestatin 0.25 m*M*, SCH 39370 0.25 m*M*. Conditions as in Fig. 1.

of MEA in vitro. The method allowed the determination of MEA, several of its metabolites, and enzyme inhibitors bestatin and SCH 39370 in the same run. ED detection was more sensitive and selective than UV detection at 205 nm for MEA and its Tyr-containing metabolites. The detection limits (S/N 3) of ED detection for MEA and its Tyr-containing metabolites were 8–12 nM (0.4–0.6 pmol per 50-μl injection), which are similar or slightly higher than those obtained with isocratic LC–ED methods [6–9]. ED detection was needed, especially in the corneal permeability studies, where the levels of permeated MEA were very low, and also in the determination Tyr and Tyr–D-Ala–Gly, since these metabolites were eluted close to UV absorbing impurities. The detection limit of MEA in UV detection (1.0 pmol) was two times higher than in ED detection. On the other hand, the linear dynamic range of UV detection extended to higher concentrations which was useful when analysing conjunctival permeability samples with high levels of MEA. UV detection was also used to determine non-electroactive enzyme inhibitors bestatin and SCH 39370 in conjunctival permeability studies, whereas in corneal permeability samples the levels of these inhibitors were close to the quantitation limits.

In the permeability studies, MEA was extensively metabolized. Tyr and Tyr–D-Ala–Gly were the major metabolites in both cornea and conjunctiva, suggesting that aminopeptidases and enkephalinase participated in the hydrolysis of MEA. The combination of an aminopeptidase inhibitor bestatin and enkephalinase inhibitor SCH 39370 increased corneal and conjunctival permeability of MEA in vitro by 11 and 5 times, respectively, with a reduction in the metabolism.

5. Conclusions

A gradient HPLC with combined UV and ED detection was a sensitive and selective method for permeability and metabolic studies of MEA in cornea and conjunctiva in vitro.

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