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Note

Reversed-phase high-performance liquid chromatography of tyrosine-containing metabolites of enkephalins using octanesulphonic acid as ion-pairing agent and radioactivity detection

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The identification and determination of the tyrosine (Tyr)-containing metabolites of enkephalin, Tyr, Tyr-Gly, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe, are of importance in studies of enkephalin degradation with different biological materials, *e.g.*, tissue homogenates, cytoplasm, blood plasma and cerebrospinal fluid. The most important enzymes involved in enkephalin degradation are aminopeptidases which cleave the Tyr-Gly bond, dipeptidylaminopeptidases which cleave the Gly-Gly bond and endopeptidases which hydrolyze the Gly-Phe bond. After incubation of the samples with ^{3}H-labelled substrate, the metabolites are usually separated by chromatography on polystyrene beads[1], followed by thin-layer chromatography and liquid scintillation counting of the separated spots[2,3]. No sensitive high-performance liquid chromatographic (HPLC) method for the separation and collection of the ^{3}H-labelled metabolites for scintillation counting has been reported. The HPLC method of Mousa and Couri[4] allows separation of the Tyr-containing metabolites in the range 10–100 ng but it is not suitable for collection of separate peaks for scintillation counting.

This paper reports a simple and rapid HPLC method for the separation and determination of the Tyr-containing metabolites of [^{3}H]enkephalins in the picogram, *i.e.*, femtomole range.

EXPERIMENTAL

[Tyr-^{3}H]Leu-enkephalin (36.6 Ci/mmol) and [Tyr-^{3}H]Met-enkephalin (30.0 Ci/mmol) were obtained from New England Nuclear (Dreieich, F.R.G.). Tyrosine, Tyr-Gly, Tyr-Gly-Gly, Leu- and Met-enkephalin were obtained from Sigma (München, F.R.G.), Tyr-Gly-Gly-Phe from Bachem (Bubendorf, Switzerland) and carboxypeptidase A from Boehringer (Mannheim, F.R.G.). As a liquid scintillation cocktail, Rialuma® from Baker Chemicals (Deventer, The Netherlands) was used.

The chromatographic system consisted of a Gynkotek high precision Model 300B solvent-delivery pump, a Rheodyne 7125 stop-flow injector, a Shimadzu SPD-2A spectrophotometric detector and an HPLC radioactivity monitor LB 504 (Berthold, Wildbad, F.R.G.). A one-channel recorder and an Apple IIe computer were used for the evaluation of the detected radioactivity. Separations were per-

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formed on a short Spherisorb ODS II column (60 mm × 4.1 mm) (particle size 3 μm). Mobile phases: buffer A, 13 m*M* citric acid, 7 m*M* disodium phosphate, 0.3 m*M* 1-octanesulphonic acid sodium salt plus methanol (90:10), pH 3.2; buffer B, 0.1 *M* potassium phosphate, adjusted to pH 6.8 by 0.1 *M* disodium phosphate, plus methanol (65:35).

The column was equilibrated with buffer A for 4 min at 4 ml/min. After application of the sample (20 μl), buffer A was used for 3 min then the system was switched to buffer B for another 5 min. The flow-rate for the elution of Tyr was 1 ml/min for the first minute and 2 ml/min thereafter for the elution of the other metabolites. The ^{3}H-labelled products were coeluted with unlabelled standards added to the sample after incubation. The column effluent was monitored with an UV detector (254 nm) and a radioactivity monitor. For the latter mode of detection the scintillation liquid was continuously mixed with the eluate and the resulting mixture passed through a flow-through cell (1 ml). After each separation the column was washed with 50% methanol for 2 min at 4 ml/min.

RESULTS AND DISCUSSION

A mixture (20 μl) of Tyr, Tyr-Gly-Gly, Tyr-Gly, Tyr-Gly-Gly-Phe, Leu- and Met-enkephalin dissolved in 1 ml of 0.1 *M* hydrochloric acid was loaded on the HPLC column, and the substances were separated as shown in the UV chromatogram (Fig. 1A).

It was possible to collect the Tyr-containing metabolites in separate fractions without any overlaps. It was very important to add the hydrophobic ion-pairing agent, octanesulphonic acid, to the mobile phase (buffer A) for the separation of Tyr-Gly-Gly and Tyr-Gly. Without this reagent, there was no separation of these two small peptides.

Eluted compounds are normally quantified by continuous UV or electrochemical detection. Electrochemical detection is suitable, because Tyr and peptides containing Tyr are electroactive[5,6], but problems can arise with endogenous tyrosine and changes in detector response when switching buffers. The UV detection method does not have the required sensitivity for measurement of small amounts of the metabolites, which are generated during incubation of biological samples with low concentrations of substrates in the nanomol range. In such cases, maximum sensitivity is obtained with radioactivity detectors.

A typical radioactivity chromatogram of the enkephalin metabolites, Tyr, Tyr-Gly, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe, and the substrates, Leu- and Met-enkephalin, obtained after incubation of an homogenate of astrocytes with added carboxypeptidase A and incubation with [^{3}H]Leu- or [^{3}H]Met-enkephalin (200 n*M*), is illustrated in Fig. 1B. The retention times (Table I) closely corresponded to those obtained with the unlabelled standards shown in the UV chromatogram.

The detection sensitivity for Leu-enkephalin and its Tyr-containing metabolites was in the picogram, *i.e.* femtomol, range. Under the conditions stated in Experimental, linear calibration plots for picogram amounts of the substrate and the metabolites were obtained (Fig. 2). The activity shown on the ordinate is the absolute number of disintegrations per minute obtained after peak integration. The background activity for the elution with buffer A was 600 dpm, that for the elution with

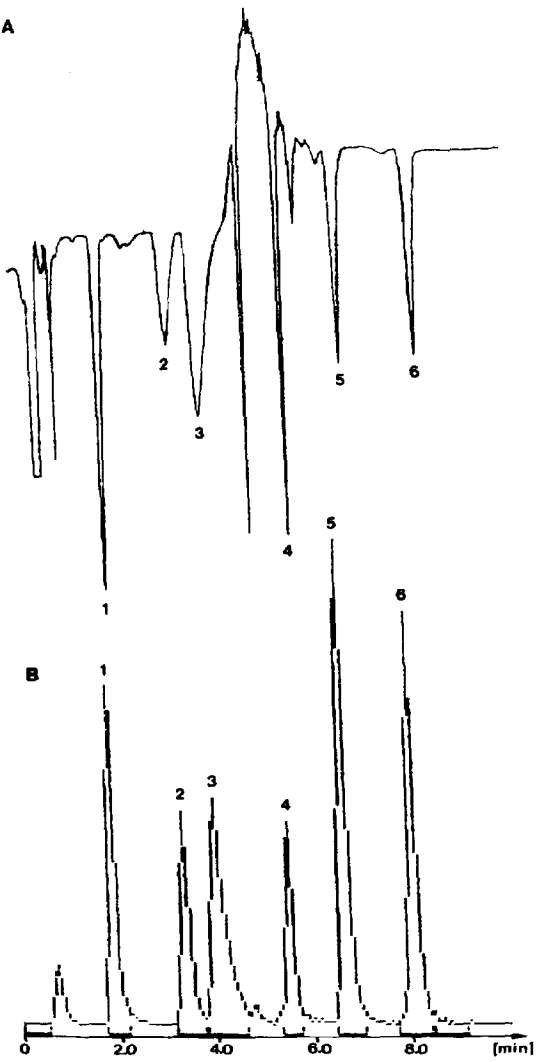

Fig. 1. Representative UV (A) and radioactivity chromatograms (B) of enkephalins and their Tyr-containing metabolites after 15-min incubation at 25°C with an homogenate of primary astrocytes from 2-day-old rats with carboxypeptidase A. The first peak represents the injection artifact, the second one (UV chromatogram) was not identified. The other peaks are Tyr (1), Tyr-Gly-Gly (2), Tyr-Gly (3), Tyr-Gly-Gly-Phe (4), Met-enkephalin (5) and Leu-enkephalin (6). The incubation mixture contained 40 μl of 10 mM dipotassium phosphate buffer, pH 7.4, 20 μl of Leu- or Met-enkephalin (200 nM), 20 μl of cell homogenate (25 μg protein), each in dipotassium phosphate buffer, and 20 μl of carboxypeptidase A suspension. Incubation was stopped with 10 μl of 1 M hydrochloric acid. After centrifugation at 8000 g, 20 μl of the supernatant were mixed with unlabelled standards, and the mixture was applied to the column. Peaks were identified by their retention times as compared to standards in the UV chromatogram. For details of the chromatography see the Experimental section.

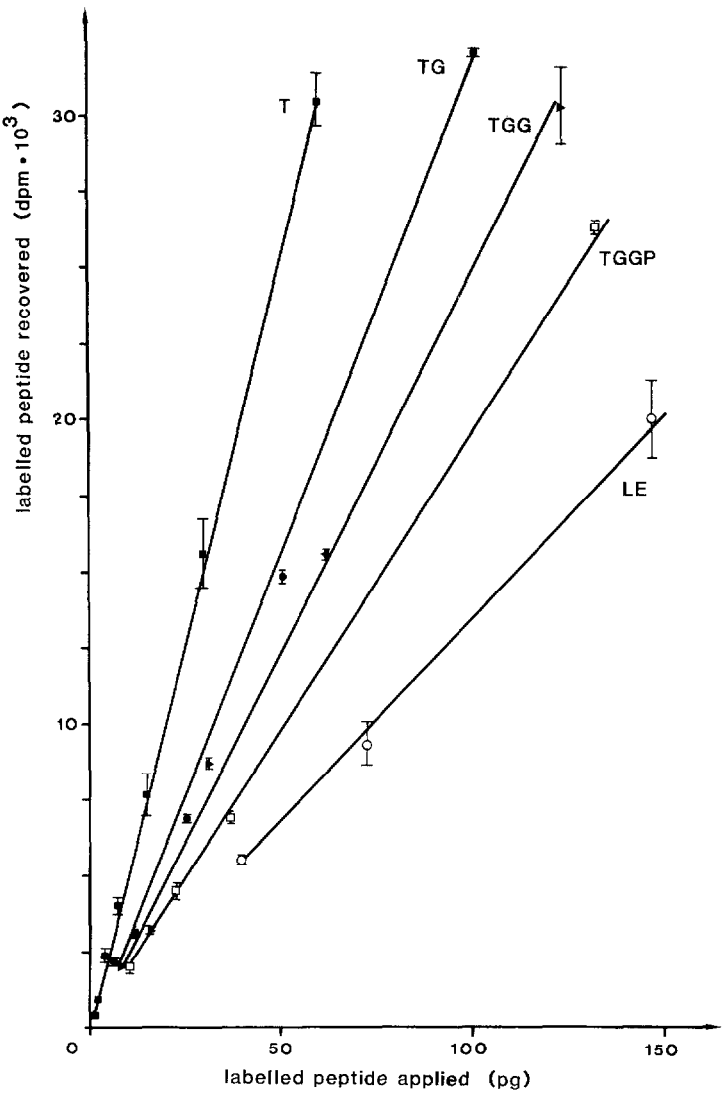

Fig. 2. HPLC-radioactivity calibration graphs for Tyr (T), Tyr-Gly (TG), Tyr-Gly-Gly (TGG), Tyr-Gly-Gly-Phe (TGGP) and Leu-enkephalin (LE). HPLC conditions as described in the text. Buffer A was used for T, TG and TGG, buffer B for TGGP and LE. The phosphate-buffered sample containing, T, TG, TGG, TGGP or LE was adjusted to 0.1 *M* with 1 *M* hydrochloric acid, protein was precipitated by centrifugation at 8000 *g* and 20 μl of the supernatant were applied to the column.

buffer B was 6300 dpm; the value was calculated for each peak region and subtracted by the slope method.

One incubated sample was analyzed on four occasions. The activities of the separated compounds eluted were highly reproducible (Table II).

For the calculation of the amounts of products, the counting efficiency and the active volume of the measuring flow-through cell (0.2 ml) must be considered. For

TABLE I

RETENTION TIMES OF ENKEPHALINS AND THEIR TYR-CONTAINING METABOLITES

Retention times are the averages from five experiments including that shown in Fig. 1.

Compound	*Retention time*
Tyr	1'56"
Tyr-Gly-Gly	3'28"
Tyr-Gly	4'08"
Tyr-Gly-Gly-Phe	5'40"
Tyr-Gly-Gly-Phe-Met	6'48"
Tyr-Gly-Gly-Phe-Leu	8'12"

TABLE II

REPRODUCIBILITY TEST

Eluted activity (dpm) of quadruplicate analysis of enkephalins and their Tyr-containing metabolites. Each compound was injected onto the column in 20 μl of 0.1 *M* hydrochloric acid and eluted (30 000 dpm $\triangleq$ 331 fmol).

Compound	*Eluted activity (dpm),* $\bar{x} \pm$ *S.D.*
Tyr	30 398 ± 613
Tyr-Gly	32 040 ± 675
Tyr-Gly-Gly	30 245 ± 1010
Tyr-Gly-Gly-Phe	115 100 ± 5975
Tyr-Gly-Gly-Phe-Met	287 330 ± 13 560
Tyr-Gly-Gly-Phe-Leu	343 000 ± 1298

this homogeneous method of adding scintillation liquid, we obtained a counting efficiency of 25% for ^{3}H. A variable quenching can be excluded, because no gradient of the mobile phase was used.

This method represents an alternative to the widely used combination of polystyrene bead chromatography[1] and thin-layer chromatography[3], and HPLC methods using an acetonitrile gradient and UV detection[4,7]. Apart from having the high sensitivity required for most biological applications, the present method substantially reduces the analysis time and allows a clear-cut separation of all Tyr-containing metabolites, particularly of Tyr-Gly from Tyr-Gly-Gly. The method has the additional advantage that the data corresponding to a separation are immediately available from the on-line detection system.

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