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STUDY OF LEUCINE-ENKEPHALIN IN RAT BRAIN BY A NEW HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

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SUMMARY

A specific method is presented for the assay of leucine-enkephalin (Leu-Enk) and its metabolites by reversed-phase high-performance liquid chromatography on a μBondapak C_{18} column with a mobile phase of methanol—water and 0.005 *M* tetrabutylammonium phosphate. The four substances are resolved by a linear program from 8 to 70% methanol in 25 min. Detection is achieved by monitoring the absorbance at 280 nm. Time of analysis can be reduced by means of a new high-speed liquid chromatography package.

This method allows the study of the effect of Phe-Ala on the cerebral metabolism of Leu-Enk. For this purpose, membrane preparations from rat striatum were incubated in the presence of [^{3}H]Leu-Enk with different concentrations of Phe-Ala from 10^{-7} to 10^{-3} *M* during 1 h at 37°C. Collected eluates were counted by liquid scintillation. The results suggest the presence of three membrane enzymes which generate the three metabolites, Tyr, Tyr-Gly-Gly and Tyr-Gly, in order of abundance. Maximum inhibition of [^{3}H]Leu-Enk degradation is obtained at a concentration of 10^{-3} *M* Phe-Ala.

INTRODUCTION

In 1975, Hughes et al. [1] isolated from pig brain two peptides with morphine-like activity at the opiate receptor level. Both of their structures corresponded to related pentapeptides differing only in the C-terminal amino acid and were accordingly named methionine- and leucine-enkephalin. Subsequently, Larsson et al. [2] demonstrated that these two peptides are localized in separate neurones in brain and intestine. Also, different studies carried out at the biological and pharmacological level suggest the occurrence of multiple types of opiate receptors: the so-called mu receptors which correspond preferentially to morphine whereas the delta receptors show a higher

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affinity for certain enkephalin derivatives, such as (D-Ala^2-D-Leu^5)-enkephalin [3]. Their different localization in various brain structures, all of them related to the cerebral cortex and limbic system, point to the possible mediation of analgesic actions by the mu receptors and emotional behaviour by the delta receptors. On the other hand, these and related peptides appear to act as neurotransmitters, being localized in specific neuronal populations where they modify neuronal activity [4] through their release upon membrane depolarization.

The identification of a mechanism responsible for the specific enzymatic inactivation of these putative neurotransmitters would provide a better knowledge of their function, especially since no specific uptake system has been found to terminate their interaction with the opiate receptors. Along these lines, it is known that both Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) can be variously cleaved by different enzymes. Soluble and membrane-bound aminopeptidases cleave the Tyr-Gly bond, carboxypeptidases work on the Phe-Met or Phe-Leu bonds whereas membrane-bound dipeptidylcarboxypeptidases, enkephalinase A [5] or ACE [6] cleave the Gly-Phe bond and a dipeptidylaminopeptidase (enkephalinase B) cleaves the Gly-Gly bond. Very recently, two membrane-bound aminopeptidases have been solubilized and characterized in rat brain [7].

Nevertheless, it is still not clear which of these enzymes, if any, may specifically inactivate both of these neuropeptides at the synaptic level. For this purpose, it would be helpful to be able to study in some detail the resulting metabolic breakdown products of the enkephalins and corresponding turnover, as well as the effects of different drugs on the activity of the responsible enzymes. This requires an analytical approach based on the isolation and unequivocal characterization of the precursor peptide and its metabolites. In this regard, high-performance liquid chromatography (HPLC) may be specially suited. The separation and analysis of a variety of neuropeptides by HPLC on reversed-phase columns has been described [8, 9]. However, no comparable work has been done on the application of these techniques to neuropeptide turnover studies, which to date have been approached through the more restrictive use of thin-layer chromatography (TLC) [10], and incomplete HPLC separation [7].

This work addresses the practical problems related to the separation of Leu-enkephalin from its metabolites; tyrosine (Tyr), tyrosine-glycine (Tyr-Gly) and tyrosine-glycine-glycine (Tyr-Gly-Gly). Due to their structural similitude, the di- and tripeptide are especially difficult to resolve chromatographically and, in fact, their separation has not been described in the literature. The significance of the separation of the parent neuropeptide and its metabolites would lie in the possibility of having access to a practical means of evaluating which of the enzymes that reportedly act on these peptides may do so specifically at the synaptic level.

The HPLC system described has been initially applied to the study of Leu-enkephalin degradation in the presence of different concentrations of the dipeptide Phe-Ala, a potent enkephalinase inhibitor.

EXPERIMENTAL

Chemicals

Tyrosine, tyrosine-glycine, tyrosine-glycine-glycine as well as leucine-enkephalin and phenylalanine-alanine were purchased from Sigma (St. Louis, MO, U.S.A.). The methanol and Pic A (commercial 0.005 *M* tetrabutylammonium phosphate solution for paired ion chromatography) were from Scharlau (Ferosa, Barcelona, Spain) and Waters Assoc. (Milford, MA, U.S.A.), respectively.

Instrumental conditions for HPLC

Leu-enkephalin was resolved from its metabolites in two different HPLC systems.

The first separations were carried out on a reversed-phase μBondapak C_{18} column (30 cm × 3.9 mm I.D., 10 μm particle size) mounted on a Model 6000 A solvent delivery system, with Model U6K universal injector and a Model 660 solvent programmer, all of them from Waters Assoc. Elution was monitored with a UV absorbance detector Model 400, also from Waters, operated at 280 nm.

Results were subsequently confirmed on a Perkin-Elmer Model 3B liquid chromatograph equipped with an LC-85 UV detector with a 2.3-μl flow-cell for high-speed HPLC [11]. Elution was also monitored at 280 nm. In this case, the column was an RP-18 (10 cm × 4.6 mm I.D., 3 μm particle size).

Chromatographic conditions

In the usual HPLC system, the four components are completely resolved by means of a 25-min linear gradient from 8 to 70% methanol. Flow-rate was adjusted to 1.5 ml/min.

In the high-speed HPLC system equipped with the 3-μm particle column, the low-strength eluent was prepared by adding 10% methanol to the 0.0005 *M* solution of tetrabutylammonium phosphate. The high-strength eluent was methanol. In this case a 4-min linear program was run from 15 to 50% of high-strength eluent at a flow-rate of 1.5 ml/min.

Preparation of the sample for HPLC

A membrane preparation of rat striatum was obtained according to the following scheme [7]. All striata of Sprague—Dawley rats were homogenized in 10 volumes of 50 m*M* Tris—HCl pH 7.4. The homogenates were centrifuged at 19,000 *g* for 20 min. The pellet was repeatedly washed with Tris—HCl buffer, resuspended and homogenized in a glass—glass potter.

The sample was centrifuged again in identical conditions as before and the pellet resuspended in 2 × 500 μl of Tris buffer and then homogenized using the same potter.

Volumes of 200 μl of the final homogenate were incubated at 37°C for 60 min in the presence of [*Tyr*-^{3}H] leucine-enkephalin and varying concentrations of Phe-Ala (10^{-7}—10^{-3} *M*).

Incubation is interrupted with 1 *M* hydrochloric acid and after centrifuga-

tion the supernatant is ready for direct injection in the HPLC system. The collected eluate fractions corresponding to the retention volumes of the peptide and the three metabolites are counted in a liquid scintillator.

RESULTS AND DISCUSSION

Fig. 1 illustrates the HPLC profile obtained using a conventional reversed-phase 10-μm particle size column. As shown, the separation of a mixture of Tyr, Tyr-Gly, Tyr-Gly-Gly and Leu-Enk is excellent but it takes more than 25 min. This, in practice, could become a disadvantage in the assay of a large series of biological samples since to the approximately 27 min of the separation one has to add the time needed for the reversed program and re-equilibration to the initial conditions, estimated at an extra 15 min per run, so that the total time comes to 42 min per injection. On the other hand, the collection of fractions for liquid scintillation counting can be substantially speeded up by resorting to a double injection of each sample, so that in the second injection Leu-enkephalin is eluted much earlier under stronger eluent conditions.

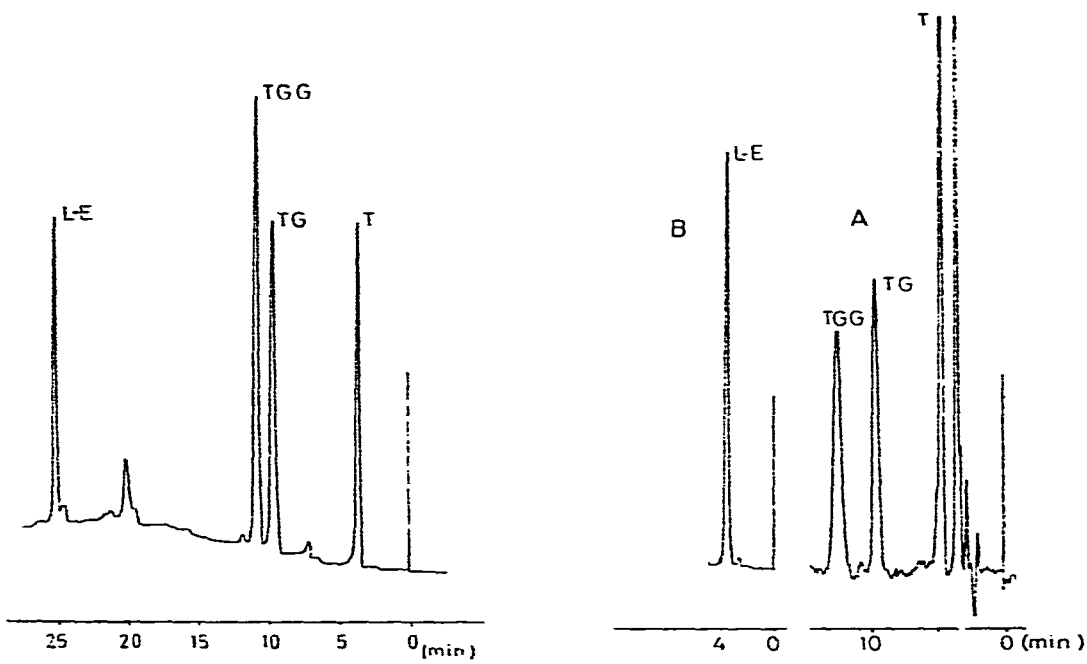

Fig. 1. HPLC profile from a sample containing known amounts of Tyr (T), Tyr-Gly (TG), Tyr-Gly-Gly (TGG) and Leu-Enk (L-E) standards. Column: μBondapak C_{18}. Linear program: 8% B to 70% B in 25 min. Flow-rate adjusted to 1.5 ml/min. Solvent A: Pic A/water. Solvent B: methanol.

Fig. 2. (A) HPLC profile from a biological sample containing added amounts of Tyr (T), Tyr-Gly (TG) and Tyr-Gly-Gly (TGG) standards. Column: μBondapak C_{18}. Isocratic conditions: Pic A/water—methanol (90:10). Flow-rate adjusted to 1.5 ml/min. (B) HPLC profile from a sample of authentic Leu-Enk (L-E). Column: μBondapak C_{18}. Isocratic conditions: Pic A/water—methanol (35:65). Flow-rate adjusted to 1.5 ml/min.

For instance, Fig. 2A shows the separation achieved injecting a biological sample spiked with Tyr, Tyr-Gly and Tyr-Gly-Gly. The eluent in this case is a mixture of Pic A with 10% methanol. Fig. 2B, on the other hand, shows the elution profile of a standard of Leu-enkephalin. The elution time has been shortened to less than 4 min by increasing the proportion of the methanol to 65%. Under these isocratic conditions (Fig. 2) the two assays can be carried out in a total combined time of 20 min, thus cutting in half the time required for the complete separation in a single injection under gradient conditions.

Consequently, a suitable scheme for the work-up of biological samples would be to divide the samples into two batches, collecting the metabolites in a first series of injections and the undegraded peptide in a second series of injections of the same samples.

The chromatographic reproducibility of retention volumes for Tyr, Tyr-Gly, Tyr-Gly-Gly and Leu-Enk, which is of importance for the appropriate collection of the corresponding eluate fractions, was calculated as 4.89 ± 0.07 ml, 13.87 ± 0.09 ml, 16.71 ± 0.21 ml and 4.57 ± 0.16 ml ($\bar{x} \pm s$, n=16), respectively.

The HPLC procedure herein described was applied to the study of the catabolism of Leu-enkephalin in rat brain in the presence of variable concentrations of Phe-Ala (10^{-7}—10^{-3} M). For this purpose, rat striatum membrane preparations were incubated with [*Tyr*-^{3}H] Leu-enkephalin and Phe-Ala. The incubates were directly injected in the liquid chromatograph and the eluent fractions corresponding to the parent peptide and metabolites were collected for radioactivity counting. The results thus obtained demonstrate the formation of three metabolites: Tyr, Tyr-Gly and Tyr-Gly-Gly. No radioactivity response could be obtained for the tetrapeptidic metabolite Tyr-Gly-Gly-Phe. Although the abundance of the three metabolites relative to the parent enkephalin varies somewhat according to the concentration of Phe-Ala, their ranking in terms of predominance does not change, as illustrated in Fig. 3.

Tyrosine, the major metabolite, invariably shows relative abundances greater than 50% in all cases, followed by the tripeptide Tyr-Gly-Gly with relative abundances of the order of 9—15%. In contrast, the dipeptide Tyr-Gly does not amount to more than 6% of the total radioactivity. Also, depending on the concentration of Phe-Ala, the recovery of unchanged Leu-enkephalin stays within 11—27%.

Another interesting observation is that these variations are not uniform across the whole concentration range of Phe-Ala. For instance, Leu-enkephalin degradation to Tyr increases when the concentration of Phe-Ala is increased from 10^{-7} M to 10^{-6} M, whereas the change from 10^{-4} M to 10^{-3} M results in less degradation with increased formation of the tripeptide. This is illustrated in Fig. 3, which depicts the individual variations of the relative amounts of each compound.

The assay of these enzymatic activities in four replicate samples run for each one of the inhibitor (Phe-Ala) concentrations shown in Fig. 3, gave coefficients of variation for the various metabolites of the order of 3—15%, reflecting an adequate intra-assay variability for biological applications.

An interesting alternative for the more rapid separation of these compounds would lie in the possibilities afforded by the modern technique of high-speed

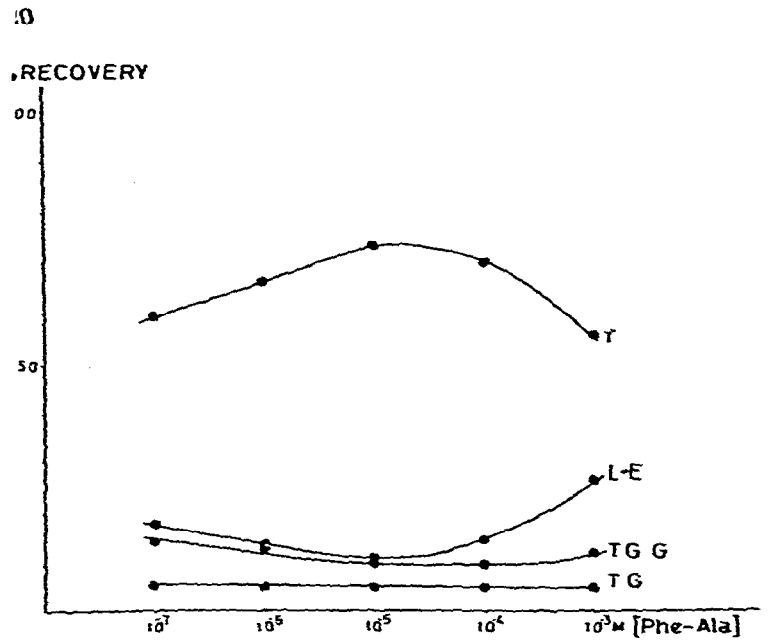

Fig. 3. Percentage recovery of Tyr (T), Tyr-Gly (TG), Tyr-Gly-Gly (TGG) and Leu-Enk (L-E) in a rat brain membrane preparation from striatum incubated with different concentrations of an enkephalinase inhibitor (Phe-Ala).

liquid column chromatography [11] carried out on specially designed instrumentation and columns of conventional diameter packed with 3-μm particles.

The results obtained by application of this new methodology to the problem posed by the separation of these four compounds are illustrated by the profile of Fig. 4. Note the substantial reduction in total analysis time, which is now of the order of 6 min plus 1 min re-equilibration of the system to initial conditions, compared to the 42 min in the conventional system; that is, a reduction of time by a factor of seven. In this manner we can inject, separate and collect the eluates corresponding to these peptides in only 7 min.

Nevertheless, the technique at present is still not free from some practical limitations like the restricted capacity of these small columns. On the other hand, this type of chromatography requires special instrumentation regarding the detector and connecting tubes in order to reduce extra column bandwidth to a level compatible with the very high efficiency of the 3—5-μm particles used to pack the columns.

The data herein presented demonstrate the convenience and speed of HPLC for the separation of Leu-enkephalin and its metabolites in biological samples. The method simplifies significantly the necessary purification process, being more specific, rapid and reliable than conventional TLC methods. Also, the individual fractions are more readily collected and counted, although presumably a radioactivity counter for TLC could also be used. Nevertheless, the speed of the HPLC method facilitates the assay of a large number of biological samples which would allow an extensive study of this particular topic in the near future.

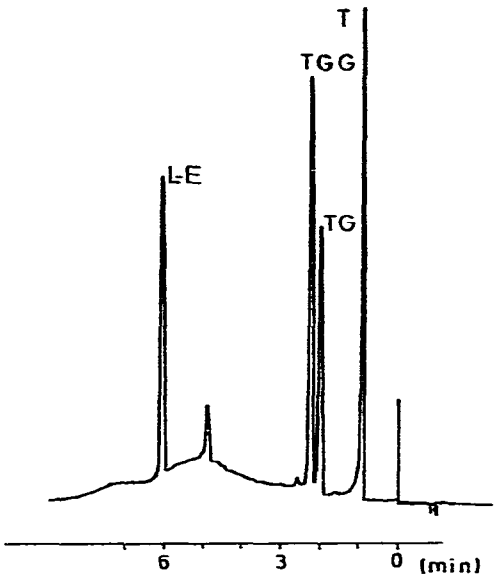

Fig. 4. HPLC profile from a sample containing Tyr (T), Tyr-Gly (TG), Tyr-Gly-Gly (TGG) and Leu-Enk (L-E) standards. Column: RP-18 (3-μm particle size). Linear program: 15% B to 50% B in 4 min. Flow-rate adjusted to 1.5 ml/min. Solvent A; Pic A/water—methanol (90:10). Solvent B: methanol.

The first results obtained in this fashion seem to confirm the absence of the tetrapeptide that would be generated by the action of a carboxypeptidase, demonstrating that the major metabolites present in these incubates are Tyr and Tyr-Gly-Gly [12]. However, the relative abundance of these two metabolites is appreciably modified by the concentration of the added Phe-Ala.

The dipeptide Tyr-Gly is always a minor component. Also, the observed predominance of Tyr indicates a greater activity of aminopeptidase compared with enkephalinase A [13].

Finally, the method is equally applicable to the study of the metabolism of Met-enkephalin since the metabolites in this case would be identical.

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