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TRACE ANALYSIS OF THE MIF* ANALOGUE PAREPTIDE IN BLOOD PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SHORT-WAVELENGTH EXCITATION FLUOROMETRY

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SUMMARY

A high-performance liquid chromatographic procedure was developed and applied to analysis of the pharmacologically active MIF* analogue pareptide in human plasma. The procedure involves formation of a fluorescent 7-chloro-4-nitrobenzyl-2-oxa-1,3-diazole (NBD-Cl) pareptide derivative followed by separation of the NBD derivative from plasma components on a 30-cm microparticle octadecylsilane bonded column. The separated derivative was quantitated using a short-wavelength excitation fluorometric detector. The detection limit of pareptide in plasma samples was 5 ng or 17 pmoles per ml of plasma. In the absence of plasma, the corresponding on-column detection limit was 0.5 pmoles.

INTRODUCTION

The analysis of a pharmacologically active peptide compound in body tissues presents a challenge to the analytical chemist; the relatively low concentrations involved and the presence of other peptides, proteins and amino acids complicate the problem. Furthermore, the peptide of interest in this study has no natural fluorescence or strong UV absorption and the only readily derivatized functional group is the secondary amine of the proline moiety. The peptide investigated was L-prolyl-N-methyl-D-leucyl-glycinamide, a synthetic analogue of MIF* [1—5]. The USAN*** name of this MIF analogue tripeptide is pareptide.

In view of the above complications, high-performance liquid chromatography(HPLC) of the fluorescent pareptide derivative was investigated. The reagent used for this purpose was 7-chloro-4-nitrobenzyl-2-oxa-1,3-oxadiazole (NBD-Cl) [6], which reacts with the prolyl secondary amino group. The

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*MIF = melanocyte stimulating hormone release-inhibiting factor.
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***USAN = United States Adopted Name.

advantages of this reagent over the frequently used dansyl chloride [7] are lower fluorescent excess reagent background and higher solubility and stability of the derivative in aqueous solution. Although the NBD derivative, unlike the dansyl derivative, can be excited in the visible range, previously reported [8] short-wavelength excitation was used since it yielded lower detection limits.

There are numerous published applications of NBD derivatization to the analysis of thiols, phenols, and primary and secondary amines [9—14]; however, a study of optimum conditions for NBD derivatization was not reported. In the present study, the yield of NBD derivatization of the prolyl-tripeptide secondary amine was investigated as a function of pH, time, and reagent excess. The optimized conditions were applied to analysis of spiked human plasma samples. A calibration curve was prepared and detection limits were determined.

EXPERIMENTAL

Materials and reagents

The MIF analogue pareptide was obtained from Ayerst Research Labs. (Montreal, Canada). NBD-Cl reagent was purchased from Regis (Morton Grove, Ill., U.S.A.). Glass-distilled acetonitrile, ethyl acetate, methanol, and pentane were purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). ACS analytical reagent grade diethyl ether, ammonium carbonate, dibasic potassium phosphate, and trichloroacetic acid were purchased from Mallinckrodt (Jersey City, N.J., U.S.A.).

Spherisorb-ODS chromatographic support (particle size 5 μm) was purchased from Spectra-Physics (Santa Clara, Calif., U.S.A.), while SAS-Hypersil was purchased from Shandon Southern Instruments (Sewickley, Pa., U.S.A.). Empty stainless-steel columns (4.1 mm I.D.) and zero-dead-volume fittings were purchased from Alltech (Arlington Heights, Ill., U.S.A.). The columns were packed in-house at 8,000 p.s.i., in an acetone slurry, with a Haskel Engineering and Supply Company (Burbank, Calif., U.S.A.) slurry packing apparatus.

Chromatographic equipment

Samples to be chromatographed were injected on column with a Valco CV-6-UHP 50-μl loop valve (Valco Instruments, Houston, Texas, U.S.A.). Chromatographic elution was monitored by a Schoeffel FS-970 fluorescence detector (Schoeffel, Westwood, N.J., U.S.A.). A Schott-Optical Glass (Duryea, Pa., U.S.A.) KV 370 cut-off filter was used to filter the fluorescence emission. Detector output was recorded with a Linear Model 385 recorder (Rainin Instruments, Brighton, Mass., U.S.A.).

Treatment of plasma prior to derivatization

Human blood plasma samples were spiked with an appropriate amount of MIF analogue pareptide to yield final concentrations ranging from 10 to 100 ng/ml. Aliquots (2 ml) of plasma samples were shaken briefly and allowed to stand for approximately 5 min. Trichloroacetic acid (100 μl of a 500 mg/ml solution) was added to each tube to precipitate the proteins. The tubes were capped, shaken for 5 min, and centrifuged at 2000 g for 25 min in an Inter-

national Equipment (Needham Heights, Mass., U.S.A.) Model UV centrifuge. The supernatant was decanted into a 10-ml screw-cap vial and extracted with two 1-ml portions of diethyl ether to remove fats and lipids. Residual diethyl ether was removed by a 2-min nitrogen flush of the solution.

Derivatization

The method used for derivatization of MIF analogue pareptide in plasma with NBD-Cl reagent is a modification of previously published methods [7, 11—14]. In general, previously published procedures [10—13] specified pH control with sodium bicarbonate or acetate buffers and derivatization temperatures ranging from 55° to 80°. In this study, saturated dibasic potassium phosphate solution, the pH of which was adjusted to 11.5 with 50% potassium hydroxide, was added to the deproteinized plasma until pH 9.5 was reached. (The volume of phosphate buffer necessary to reach pH 9.5 can be predetermined, thus simplifying the procedure.) One volume of pH 9.5 plasma was then mixed with two volumes of an acetonitrile solution of NBD-Cl reagent (0.5 mg/ml).

Due to high concentration of buffer in the aqueous phase, the water—acetonitrile mixture yields a two-phase system. Since most of pareptide is in the water phase while the NBD reagent is in the acetonitrile phase, derivatization was facilitated by continuous tumbling of capped vials in a 50° water bath for 40 min. A rotating wheel device was used for this purpose.

The vials were then removed from the bath and acetonitrile was evaporated by a stream of nitrogen. To reduce the overload of the reversed-phase column and prolong its life, the aqueous phase of the derivatized plasma solution was extracted with one 2-ml aliquot of pentane—diethyl ether (1:1) solution, which removed some of the NBD derivatives less polar than the NBD derivative of MIF analogue pareptide. The remaining partially purified aqueous phase was extracted with three 2-ml aliquots of ethyl acetate and the organic layers were combined. This procedure was carried out in a centrifuge tube so that the samples could be spun (1 min at 1000 g) to yield better separation of the aqueous and organic phases. The ethyl acetate fraction was then blown to dryness with a stream of nitrogen and the residue was dissolved in the chromatographic solvent (1 or 2 ml, depending on the amount of pareptide NBD derivative).

The above procedure was simplified when pareptide was in water rather than plasma solution. The acetonitrile solution of the NBD reagent was added to pH 9.5 aqueous solution of pareptide and the pareptide was derivatized as described. After the reaction, acetonitrile was blown off and the remaining aqueous phase was appropriately diluted with the chromatographic solvent.

Chromatography

The analytical system employed to generate the calibration curve was a combination of two 15 cm × 4.1 mm I.D. columns of Spherisorb-ODS and a 5 cm × 4.1 mm I.D. pre-column of Hypersil-SAS connected in series. The injection loop size was 50 μl. The chromatographic solvent system was a mixture of aqueous ammonium carbonate (200 mg/l)—methanol—acetonitrile (60:25:15, v/v) and was pumped at a flow-rate of 1.0 ml/min. Samples were

injected with the 50-μl loop valve, which was flushed with chromatographic solvent between each sample injection. Fluorometric detector settings were as follows: excitation at 220 nm, KV 380 nm emission filter, 0.02 μa full-scale expansion fluorometric response range, sensitivity 4.3, and a time constant of 8.0 sec. The NBD derivative of pareptide had a retention time of ca. 23 min.

RESULTS AND DISCUSSION

Although the NBD-Cl reaction and its derivatization conditions have been previously reported [7, 10—14], analysis of pareptide in blood plasma necessitated further refinement of the reaction variables. Thus it was ascertained that pH 9.5 and a large (5000-fold) excess of the NBD-Cl reagent are necessary for optimum pareptide derivative yield in the presence of blood plasma constituents. It was also established that at 50° the optimum reaction time is ca. 50 min.

However, even under optimum reaction conditions, the apparent NBD-Cl reaction yield in blood plasma was 50—60% of the yield obtained in the absence of blood plasma constituents. To correct for the incomplete reaction yield, quantitation of the pareptide in blood plasma was based on a calibration curve obtained by spiking blank plasma with known amounts of pareptide.

Fig. 1 depicts the calibration curve observed in the 20—100 ng of pareptide per ml of plasma concentration range. The observed linearity of the curve and zero intercept indicate that the blank response is negligible and the percentage yield is constant in the effective concentration range. The linear relationship between the fluorometric detector response and concentration of pareptide in the spiked blood plasma also indicates that the NBD-Cl reagent is present in a sufficient excess. Negligible blank plasma response may be attributed to the selectivity of the chromatographic system.

The chromatographic system selected for the separation and quantitation of pareptide NBD derivative involved reversed-phase chromatography on octadecylsilane (ODS) bonded support and detection with a fluorometric detector at a short excitation wavelength. The choice of the chromatographic system was based on the reproducibility of retention times, capacity of the support, and compatibility of the derivatized sample solution with the mixed aqueous—organic chromatographic solvent. Although an adsorptive support and organic chromatographic solvent system could also be used, adsorptive column supports usually have more limited column life and thus are subject to changing retention times and decreasing column efficiency. In view of the relative complexity of plasma extracts and presence of many relatively polar compounds, chromatography on adsorptive column supports was not investigated.

Selection of fluorometric detection was dictated by two considerations: inherent sensitivity of short excitation wavelength fluorescence [8] and the large excess of NBD reagent. Since only NBD derivatized tripeptide fluoresces, partial overlap between excess NBD reagent and NBD derivative of pareptide does not impair fluorometric quantitation.

Fig. 2 illustrates chromatograms of representative blank and spiked extracts of NBD derivatized human plasma extracts. Since the spiked sample contained 20 ng of pareptide per ml of plasma, the apparent detection limit is about 5 ng
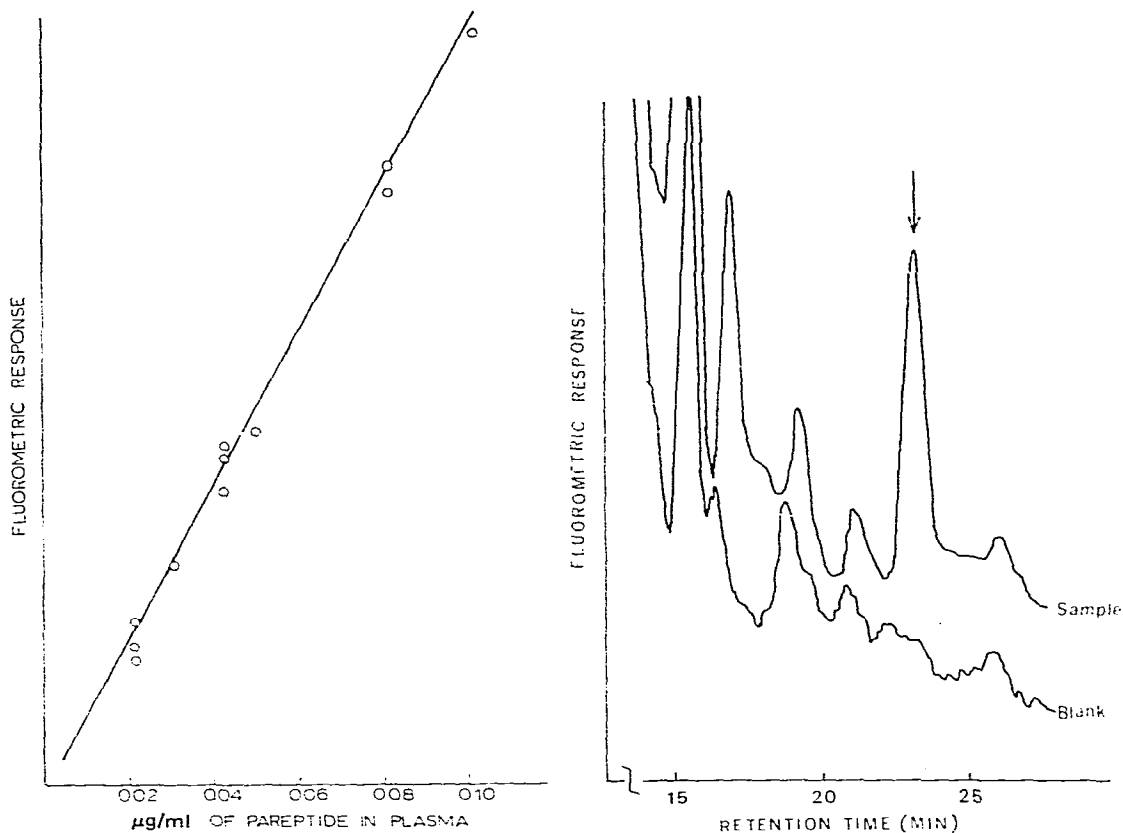
Fig. 1. Calibration curve obtained with human plasma containing variable amounts of pareptide.

Fig. 2. HPLC chromatograms of a blank plasma and a plasma sample containing pareptide at 20 ng/ml concentration; arrow identifies pareptide peak in the spiked plasma sample.

per ml of plasma. This limit involves an on-column injection of about 0.5 ng, which is equivalent to about 1.7 pmoles of tripeptide. Additional enhancement of sensitivity is possible if the chromatographic solvent is modified to yield a shorter retention time and consequently a sharper peak for the NBD pareptide derivative. However, relatively high background at short retention times requires chromatographic clean-up of the derivatized plasma extract before the analytical column solvent system can be modified. Since chromato-graphic clean-up of the derivatized plasma extract would significantly lengthen the procedure, it was not used in this study.

Fig. 3 illustrates chromatograms of pareptide derivatized in the absence of plasma and chromatographed using a solvent system which yielded a retention time of 8 instead of 22 min. The apparent detection limit under these conditions is about 0.5 pmoles which is almost 10 times more sensitive than the detection limit reported in the literature [41] for an NBD derivative of proline. The observed discrepancy may be attributed to the hemi-spherical mirror incorporated in the detector flow-cell and short excitation wavelength used in this

study. The latter contribution may be explained in terms of a relatively high electronic absorption band intensity of the singlet—singlet $\pi \to \pi^*$ transition which is promoted by the short-wavelength excitation [15]. The previously reported study [14] involved longer excitation wavelength and subsequently lower band intensity.

Although the observed 0.5-pmole detection limit for the NBD pareptide derivative in the absence of plasma is about five times less sensitive than the detection limit of previously reported [8] dansyl pareptide derivative, NBD derivatization was found to be more suitable for analysis of pareptide in the presence of plasma. Relative instability and high background of plasma derivatized with dansyl reagent offset its higher sensitivity advantage.

Since it is conceivable that some patient plasma samples could contain significant amounts of closely related tripeptides (e.g., natural MIF tripeptide) which could interfere significantly with the detection and quantitation of MIF analogue tripeptide, a mixture of pareptide and natural MIF tripeptide-NBD derivatives was chromatographed. Although the two tripeptide compounds have similar molecular structure, a complete separation of the two NBD-tripeptide derivatives was observed (Fig. 4). As expected, natural MIF tripeptide has a shorter retention time since it is a more polar compound than pareptide.
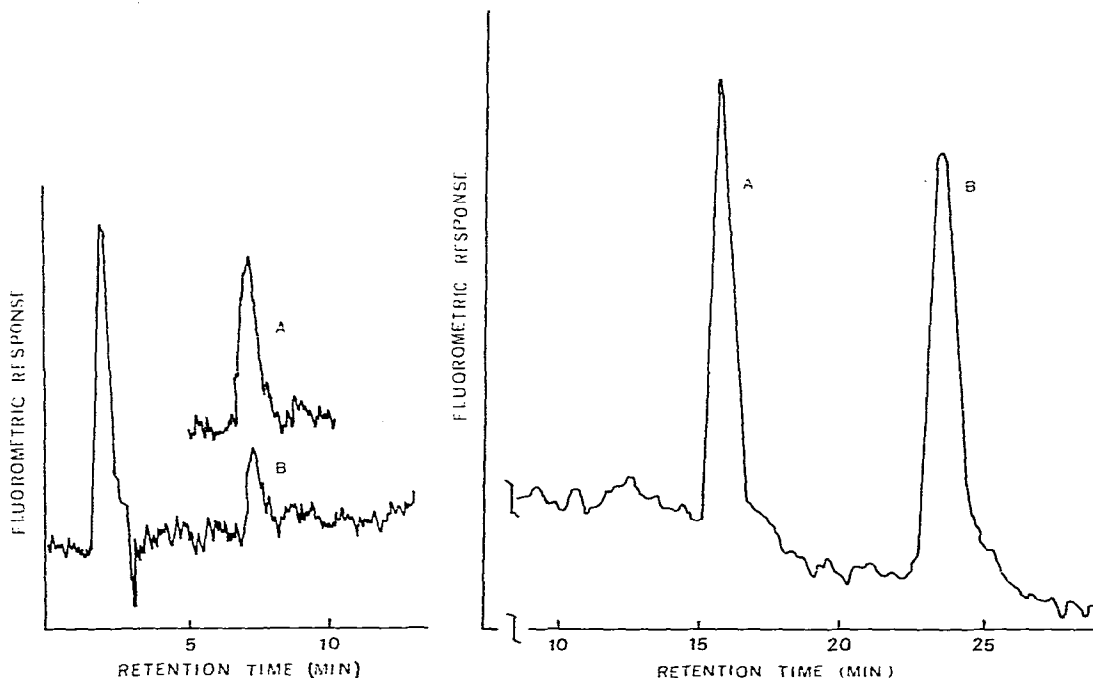

Fig. 3. HPLC chromatograms of NBD pareptide derivatives formed and chromatographed in the absence of plasma; scans A and B represent 0.4-ng and 0.2-ng on-column injection peaks of pareptide, respectively.

Fig. 4. HPLC chromatogram of the natural MIF tripeptide (A) and pareptide (B) NBD derivatives.

Subsequently, the natural MIF tripeptide cannot be used as an internal standard in our procedure since its peak is in an area where large excipient peaks are usually observed. However, an internal standard is not necessary in this procedure since no bias and a linear calibration curve (Fig. 1) were observed.

CONCLUSIONS

In conclusion, an NBD derivative of the MIF analogue pareptide was separated from plasma constituents by a HPLC reversed-phase system. As was reported in a previous publication [8], the fluorometric detection limit was enhanced by the use of short-wavelength excitation. In the presence of plasma excipients the apparent detection limit of pareptide is 5 ng per ml of plasma. Further enhancement of method sensitivity is possible with additional plasma extract clean-up which could allow shorter retention time for the pareptide NBD derivative and consequently enhanced peak height. Another method refinement which could increase the accuracy of the procedure involves use of an internal standard. However, the calibration curve obtained with spiked human plasma samples indicates that the present procedure can quantitate pareptide in human plasma samples in the low nanogram (or picomole) concentration range without the aid of an internal standard.

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