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DETERMINATION OF TRANEXAMIC ACID IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING SELECTIVE PRE-COLUMN DERIVATIZATION WITH PHENYL ISOTHIOCYANATE

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SUMMARY

A high-performance liquid chromatographic method for determination of tranexamic acid in human serum using a selective derivatization has been developed. Tranexamic acid in the sample was allowed to react with phenyl isothiocyanate to form the phenylthiocarbamoyl derivative. Interfering α-amino acids in the sample were eliminated by selective derivatization to phenylthiohydantoin derivatives by acid treatment of the phenylthiocarbamoyl derivatives followed by solvent extraction. Then, the sample was analysed by conventional high-performance liquid chromatography with ultraviolet detection. The limit of detection of this method for serum sample was 0.2 μg/ml at a signal-to-noise ratio of 2. This method gave results comparable with those obtained by amino acid analysis (regression line: $y = 0.4531x - 0.02596$, $r = 0.9998$, $n = 21$).

INTRODUCTION

Tranexamic acid (TA), *trans*-4-aminomethylcyclohexane-1-carboxylic acid, is a potent and specific inhibitor of fibrinolysis [1]. It has been widely used as an antiplasmin drug for almost twenty years, and its effectiveness in the treatment of various fibrinolytic disorders is well established. Therapeutic drug monitoring is essential for adequate medication, and thus a simple method for determining TA in biological fluids is required. Methods used to date have included gas chromatography [2], gas chromatography–mass spectrometry [3] and amino acid analysis [4], and high sensitivity and high selectivity have been achieved. However, these methods are all complicated and expensive. More recently, other methods using high-performance liquid chromatography (HPLC) with fluorimetry have been reported [5–7], but two of these have some difficulties in determining serum concentrations below 1 μg/ml, and details of the third are not

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available. This paper describes a new HPLC method using phenylthiocarbamoyl (PTC) derivatization, a selective clean-up procedure and conventional HPLC with UV detection (254 nm).

EXPERIMENTAL

Chemicals and reagents

TA and *cis*-4-aminomethylcyclohexanecarboxylic acid (*cis*-AMCHA) were synthesized by Daiichi Seiyaku (Tokyo, Japan). DL-β-Amino-*n*-butyric acid, DL-β-aminoisobutyric acid, 5-amino-*n*-valeric acid, 3-aminocyclohexanecarboxylic acid and phenyl isothiocyanate (PITC) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and 5-sulphosalicylic acid dihydrate was from Pierce (Rockford, IL, U.S.A.). 4-Aminomethylbicyclo[2.2.2]octane-1-carboxylic acid (AMBOCA) was gift from KabiVitrum (Uxbridge, U.K.). Other chemicals used were of analytical grade.

HPLC apparatus and chromatographic conditions

HPLC analysis was performed using a Model 655A-11 liquid chromatograph (Hitachi, Tokyo, Japan), equipped with a sample-injection valve fitted with a 20-μl loop (No. 7125, Rheodyne, Cotati, CA, U.S.A.) and Model 655A variable-wavelength UV detector (Hitachi). The system was equipped with a stainless-steel column (15 cm $\times$ 4.6 mm I.D.) packed with 5-μm octyl silica (Cosmosil $5C_8$, Nakarai Chemicals, Kyoto, Japan). The mobile phase was of a mixture of 20 m*M* phosphate buffer (pH 7.0)–ethanol (90:10, v/v) and the flow-rate was 1.8 ml/min. Isocratic elution was carried out at 23–25°C. The eluate was monitored at 254 nm.

Sample preparation

Human serum was obtained from Flow Labs. (Melean, VA, U.S.A.) and used as blank serum after being tested for the absence of TA by amino acid analysis (see below). Human serum samples were collected from a healthy volunteer (Japanese, male, aged 38 years) just before and at 0.25, 0.5, 1, 2, 3, 4, 6, 8, 24 h after the oral administration of 0.5 g of TA in syrup. Informed consent was obtained from him, and the experiment was conducted by medical staff under the supervision of a doctor. The serum samples were stored frozen (−20°C) until required for analysis.

To 0.5 ml of serum in a 10-ml glass centrifuge tube, 5 μl of a 58.4 μg/ml solution of internal standard (I.S.; 3-aminocyclohexanecarboxylic acid) were added and mixed well by swirling. Then, 2 ml of ethanol were added to the serum and the contents was mixed on a vortex mixer. The tube was centrifuged at 1500 *g* for 10 min, and the supernatant was transferred to another centrifuge tube. To the supernatant, 1 ml of 10 m*M* borax solution (pH 9.2) and 13 μl of PITC were added and kept at 40°C for 30 min. The resulting mixture was extracted three times with 2-ml portions of xylene and centrifuged (1500 *g*, 10 min, 23–25°C). The organic layer was discarded and the aqueous layer was acidified with 1 ml of 35% (w/w) hydrochloric acid. The solution was heated at 80°C for 10 min, then evap-

orated to dryness under reduced pressure. The residue was dissolved in 1 ml of 0.1 *M* borax solution and extracted twice with 2-ml portions of benzene. The organic extracts were combined and evaporated to dryness under nitrogen. The residue was redissolved in 0.5 ml of mobile phase, and a 10-μl portion was injected into the chromatograph.

Calibration curve

The standard samples were prepared by dissolving TA in blank human sera at concentrations of 0.022, 0.055, 0.11, 0.45, 1.13, 2.82, 5.63 and 11.26 μg/ml. A 0.5-ml portion of the standard sample at each concentration was processed as described in *Sample preparation*. A calibration curve was obtained by plotting the peak-height ratios (TA to I.S.) against the concentrations of TA in the standard samples. The standard curve for urine was prepared in the same way as that for serum.

Preparation of phenylthiocarbamoyl tranexamic acid (PTC-TA)

In 40 ml of pyridine, 810 mg of TA and 2.4 ml of PITC (2.71 g) were dissolved and allowed to stand for three days at 23–25 °C. A 10-ml portion of distilled water was added to the reaction mixture, which was then extracted with 40 ml of benzene. The extract was shaken with 10 ml of 1 *M* sodium hydroxide solution, and the aqueous layer was separated after standing. The aqueous layer was transferred to an other flask and titrated with 2 *M* hydrochloric acid. The gummy oil that was precipitated at first was removed with a spatula. By further acidification, white fine crystals were precipitated. The crystals were collected by filtration, washed with distilled water and dried for 20 h over phosphorus pentoxide under vacuum to give 230 mg of PTC-TA. The melting point of the crystals was 172–174 °C, and analysis of the compound confirmed the chemical structure and its purity. Calculated for $C_{15}H_{20}O_2N_2S$: C, 61.63; H, 6.90; N, 9.58; S, 10.90%. Found: C, 61.59; H, 6.85; N, 9.62; S, 10.87%.

The chemical structure of the reaction product was also supported by the electron-impact mass spectrum and the ^{1}H NMR spectrum.

Chromatographic conditions

Using authentic PTC-TA, which was prepared as described above, chromatographic conditions for the analysis of PTC-TA were studied. We tried to develop suitable chromatography for PTC-TA analysis using octadecylsilica columns, because they are widely used. However, despite many tests with a Nucleosil $5C_{18}$ column, we were unable to find suitable conditions for the determination of TA because of the considerable tailing of PTC-TA. Therefore, the column was replaced by an octylsilica column, Cosmosil $5C_8$, and PTC-TA was then eluted as a sharp peak. Two mobile phases were found to elute PTC-TA as a sharp peak: the mixture described in the Experimental section and a mixture of acetate buffer (0.1 *M*, pH 4) and acetonitrile (75:25, v/v). We adopted the former because it eluted fewer UV-absorbing materials near the retention time of PTC-TA.

Internal standard

Four chemically synthesized amino acids (Table I), which were easily available from commercial sources, were chosen as candidates for the I.S. and their chromatographic properties were examined. All of their PTC derivatives gave sharp and symmetrical peaks on the chromatogram. By comparing the retention times of these derivatives (Table I) with that of TA, 3-aminocyclohexanecarboxylic acid was adopted as I.S. A typical chromatogram of the mixture of PTC derivatives of TA and I.S. is shown in Fig. 1. The relationship between the concentration of TA and the peak-height ratio of TA to I.S. was examined as follows. To 0.5 ml of TA solution in water, a given amount of the I.S. was added. The sample was processed as described above in *Sample preparation*, except that the hydrochloric acid treatment and subsequent extraction were omitted. The peak-height ratios correlated well with the concentrations of TA over the range 0.11–40 μg/ml and regression analysis gave 0.9995 as the correlation coefficient (equation of line: $y=0.4273x+0.0258$).

TABLE I

AMINO ACIDS TESTED AS INTERNAL STANDARD AND RETENTION TIMES OF THEIR PHENYLTHIOCARBAMOYL DERIVATIVES

Column, Cosmosil $5C_8$, 15 cm × 4.6 mm I.D.; eluent, 20 m*M* phosphate buffer (pH 7.0)–ethanol (90:10, v/v), flow-rate, 1.8 ml/min.

Amino acid	Retention time of PTC derivative (min)
DL-β-Amino-*n*-butyric acid	4.5
DL-β-Aminoisobutyric acid	4.5
5-Amino-*n*-valeric acid	9.0
3-Aminocyclohexanecarboxylic acid	14.1
Tranexamic acid	10.8

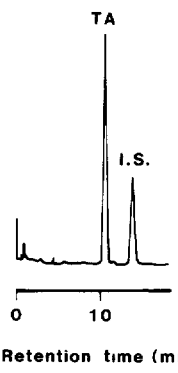

Fig. 1. High-performance liquid chromatogram of tranexamic acid (TA) and the internal standard (I.S.) converted into their phenylthiocarbamoyl derivatives.

Determination of TA by amino acid analysis

The method of Sano et al. [4] was used in this study with slight modification. To 1.0 ml of serum in a glass centrifuge tube, 10.6 μg of AMBOCA were added as internal standard. Then 0.3 ml of a 20% (W/V) solution of 5-sulphosalicylic acid was added and mixed on a vortex mixer. The tube was centrifuged at 1500 *g* for 10 min at 23–25°C. The supernatant was separated, and a 50-μl aliquot was analysed by amino acid analyzer (Model 835, Hitachi). Analysis was performed on a column (15 cm × 2.6 mm I.D.) packed with strongly acidic cation-exchange resin (No. 2619, Hitachi) by two-step elution. The column was heated at 53°C. Eluting solvents were 0.12 *M* citrate buffer (pH 4.9, Na^+ 1.2 *M*) in step 1 (start to 16 min) and 0.12 *M* citrate buffer (pH 6.7, Na^+ 0.35 *M*) in step 2 (thereafter), respectively. The flow-rate was 13.5 ml/h. Ninhydrin reagent was used for detection. The retention times for TA and AMBOCA were 28 and 32 min, respectively.

RESULTS

Selectivity

A 0.5-ml aliquot of blank human serum was processed as described in Experimental, except that the hydrochloric acid treatment and subsequent extraction were omitted. The chromatogram (Fig. 2) indicates that human serum contained many components that could be labelled with PITC, and that these components severely interfered with chromatographic analysis of TA. These interfering components were not removed by extraction with any organic solvent examined.

After deproteinization and reaction with PITC, the serum sample was heated with hydrochloric acid and then extracted with organic solvent in basic conditions as described in Experimental. The chromatogram of blank serum obtained

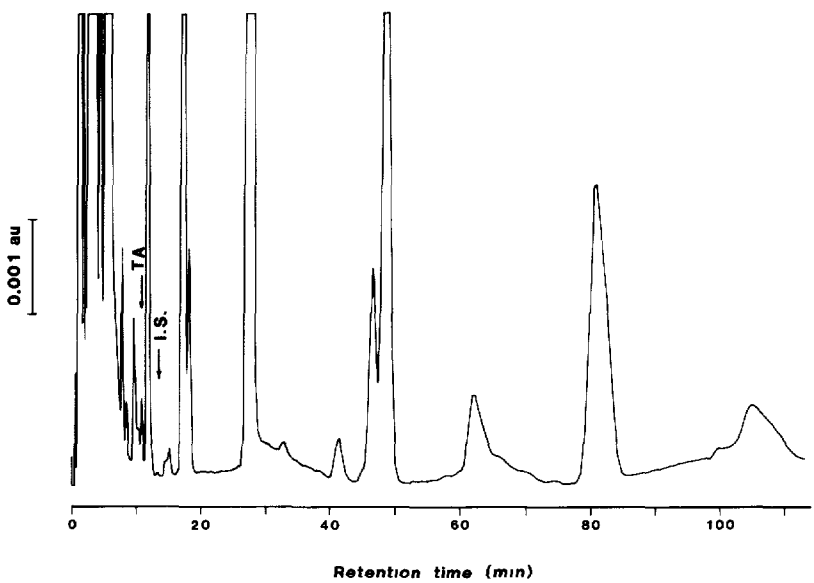

Fig. 2. High-performance liquid chromatogram of blank serum treated with PITC.

by this procedure is shown in Fig. 3A. Almost all the peaks that were eluted in a wide range of retention time, as indicated in Fig. 2, were extensively eliminated, and no interfering materials were eluted at the retention times of the TA and I.S.

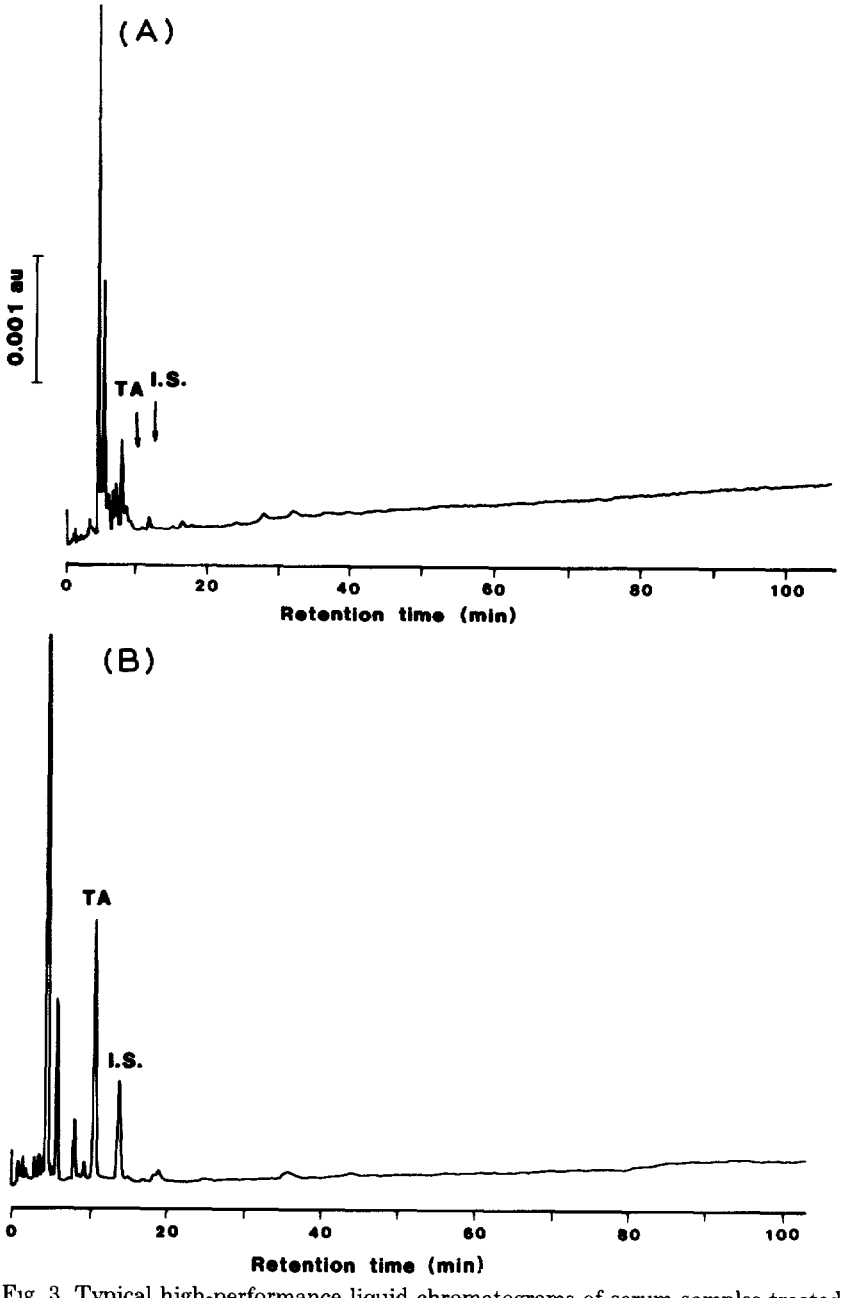

Fig. 3. Typical high-performance liquid chromatograms of serum samples treated with PITC and heated under acidic conditions to generate PTH derivatives. (A) Blank serum; (B) one of the serum samples for standard graph, TA = 22.52 μg/ml.

TABLE II

ACCURACY AND PRECISION OF THE PROPOSED METHOD FOR DETERMINATION OF TRANEXAMIC ACID IN SERUM

Concentration (μg/ml)		Accuracy [(found/added) × 100] (%)	Precision, C.V. (%)
Added	Found (mean ± S.D.)		
Intra-assay (n = 10)			
21.15	21.16 ± 0.98	100.1	4.6
4.23	4.17 ± 0.08	98.5	1.9
0.53	0.58 ± 0.04	112.9	5.7
Inter-assay (n = 5)			
21.15	22.12 ± 1.66	104.6	7.9
4.23	4.28 ± 0.10	101.1	2.4
0.53	0.59 ± 0.03	116.9	7.2

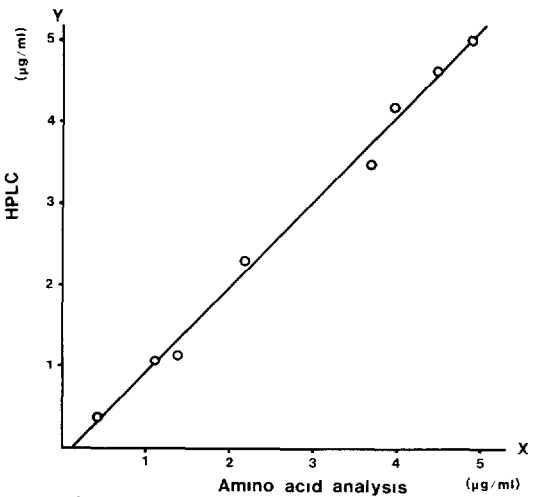

Fig. 4. Correlation between concentrations of tranexamic acid in human serum determined by HPLC and by amino acid analysis: $y = 1.053x - 0.1356$, $r = 0.9965$.

derivatives. A typical chromatogram of a serum sample spiked with TA and I.S. is shown in Fig. 3B. The peak heights of TA and I.S. could be measured precisely without any difficulty. The peak of *cis*-AMCHA, the stereoisomer of TA, was not detected at the corresponding retention time, (17 min), and no evidence of isomerization was observed.

Linearity, recovery and precision

Human blank serum was spiked with various amounts of TA and a given amount of I.S., and processed as described in Experimental to produce a standard curve. A linear relationship was observed in the range 0.22–22.5 μg/ml (equation of line: $y = 0.4531x - 0.02596$, $r = 0.9998$). The relative recovery and the precision, defined as the coefficient of variation (C.V.), are summarized in Table II. The relative recoveries were 100.1, 98.5 and 112.9% at concentrations 21.15, 4.23 and

0.53 μg/ml, respectively. The C.V. values were less than 6% intra-assay and less than 8% inter-assay. Absolute recovery was 34.8 ± 1.9% (mean ± S.D.) at a serum concentration of 5.6 μg/ml.

Accuracy

To confirm the accuracy of the method, the concentrations determined were compared with those obtained by amino acid analysis for the same serum samples. The serum samples were obtained from a healthy volunteer who received orally 0.5 g of TA. Fig. 4 shows that concentrations of TA determined by the two methods are highly correlated, with a small intercept and the slope of the regression line close to unity. Thus, these two methods give comparable results for the analysis of TA in human serum.

DISCUSSION

It is necessary to derivatize TA into a chromophore, because it has no functional group that absorbs UV radiation. Many kinds of derivatization reagents applicable to the determination of amino acids or oligopeptides have been reported [8]. Among these, PITC was considered to be suitable for a labelling reagent, since it is inexpensive and its reaction proceeds quantitatively even in aqueous conditions. Moreover, the reaction conditions for amino acids and peptides have been thoroughly studied [9–12]. Heinrikson and Meredith [11] have reported that both octylsilica and octadecylsilica columns gave good results in the analysis of PTC-amino acids. Scholze [12] has also shown that an octadecylsilica column could separate PTC-amino acids effectively. In the present study, however, we could not find suitable conditions for the analysis of PTC-TA on octadecylsilica columns, possibly because TA is more lipophilic than common α-amino acids. Therefore, we determined suitable conditions using an octylsilica column with a mobile phase of 20 m*M* phosphate buffer (pH 7.0)–ethanol (90:10, v/v).

The compound used as I.S. should be similar to TA in chemical properties and should not occur in biological fluids. For these reasons, 3-aminocyclohexanecarboxylic acid was adopted as I.S. in this method. This compound could exist in two diastereomeric forms, because it has two chiral centres at the 1- and 3-positions in its cyclohexane ring, and hence could produce two peaks in chromatographic analysis. However, the compound that was purchased gave only one peak; we later discovered this to be the *cis*-isomer.

The chromatogram shown in Fig. 2 indicates that human serum contains many components that can be labelled with PITC, and that these components severely interfere with HPLC analysis of TA. Since these interfering components were not extracted with any organic solvent examined, they were considered to be highly polar compounds. Accordingly, it seems reasonable to assume that free α-amino acids in the serum may be responsible for this kind of interference, because it is well known that serum contains many free amino acids in appreciable concentrations [13]. These results stimulated us to devise a new derivatization procedure for removing the interference of the endogenous free amino acids. In this regard, it has been well established that PTC derivatives of α-amino acids can be con-

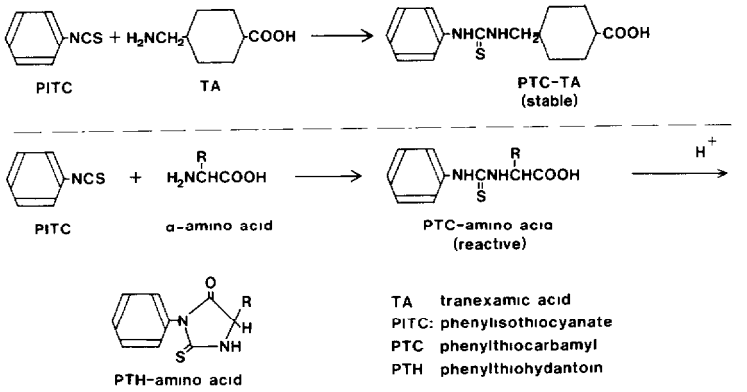

Fig. 5. Reaction procedure for derivatization of tranexamic acid and α-amino acids.

verted into PTH derivatives under acidic conditions, as illustrated in Fig. 5, and the PTH derivative loses the carboxyl group to afford the non-ionizable compound, which is extractable with organic solvent even in basic conditions [9]. On the contrary, it is conceivable that PTC derivatives of TA and I.S. do not undergo this cyclization reaction and therefore they remain as carboxylic acids, because the carboxyl groups in these derivatives are too far from the amino groups to form the thiohydantion ring. This difference in chemical properties between PTC derivatives of TA and I.S. and those of α-amino acids was considered to explain the successful removal of interfering materials from the samples. The present results show that the interfering materials are almost completely eliminated by the acid treatment of PTC-derivatized human serum followed by extraction, as indicated in Fig. 3.

Miyazaki et al. [3] have reported the isomerization of TA to *cis*-AMCHA under acidic conditions during derivatization. The same kind of side-reaction would decrease the absolute recovery of PTC-TA and compromise the sensitivity of the method. However, the peak of TA was not accompanied by that of *cis*-AMCHA, as indicated in Fig. 3B, indicating that this isomerization does not occur under the conditions used in this study.

All previous methods for the determination of TA need expensive and sophisticated apparatus and skillful technique to measure the concentration of the drug below 1 μg/ml in serum. However, the results of this study show that analysis of TA without expensive apparatus is possible. The present method needs only reaction with PITC, followed by extraction and analysis with an isocratic HPLC system in which the PTC derivative of TA can be detected by absorption at 254 nm.

This unique clean-up technique, which consists of selective derivatization of α-amino acids to PTH derivatives and extraction, might be also applicable to the analysis of other biologically important amino acids, such as γ-aminobutyric acid, baclofen and ε-aminocaproic acid, which are not α-amino acids.

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