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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF FREE HYDROXYPROLINE AND PROLINE IN BLOOD PLASMA AND OF FREE AND PEPTIDE-BOUND HYDROXYPROLINE IN URINE

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SUMMARY

A rapid, accurate and sensitive method for the determination of free hydroxyproline and proline in plasma and of total hydroxyproline in urine has been developed. Free imino acids and internal standard are extracted from plasma by trichloroacetic acid precipitation of protein and they are selectively derivatized with 4-chloro-7-nitrobenzofurazan, after reaction of the acid extract with *o*-phthalaldehyde. The highly fluorescent adducts of imino acids are separated on a Spherisorb ODS 2 reversed-phase column using acetonitrile—0.1 *M* sodium phosphate buffer, pH 7.2 (9:91, v/v) as mobile phase, followed by fluorometric detection. Total hydroxyproline determination in urine hydrolysates is carried out by reaction of the imino acid with 4-chloro-7-nitrobenzofurazan after clean-up on a Sep-Pak C_{18} cartridge of the *o*-phthalaldehyde-treated sample, high-performance liquid chromatographic separation and fluorometric quantitation of the derivative.

INTRODUCTION

In liquid chromatography post- and pre-column derivatization with fluorogenic reagents is increasingly being used to improve the detection of amino acids in biological material. In this field, the pre-column derivatization of amino acids with *o*-phthalaldehyde (OPA)—mercaptoethanol reagent, followed by high-performance liquid chromatographic (HPLC) separation and

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fluorometric detection of the derivatives, appears at present to be the most reliable method [1—3]. However, this procedure has the disadvantage that proline (Pro) and hydroxyproline (Hyp) do not give a positive reaction with OPA. The selectivity of this reagent toward the primary amino group can be extended to imino acids by using post-column derivatization after chemically changing a secondary amino group into a primary one by treatment with hypochlorite [4—6]. Nevertheless, these procedures are not suitable for the simultaneous analysis of all amino acids since complex apparatus is required and they are so time-consuming that their use is restricted to small series of determinations. Recently, the reagent 4-chloro-7-nitrobenzofurazan (NBD-Cl) has been proposed for the chromatographic determination of primary and secondary amines [7—9]. This reagent has proved to be effective for Hyp and Pro determination in plasma [7] using post-column derivatization after chromatographic separation on a cationic exchange resin. Moreover, Bellon et al. [10] used NBD-Cl for the determination of 4-Hyp and 3-Hyp in urine by thin-layer chromatography of the derivatives. These methods, however, are time-consuming and tedious when compared with the results that can be achieved by HPLC with the pre-column derivatization procedures.

This paper deals with the development of an HPLC assay which allows the easy determination of free Hyp and Pro in blood plasma and of free and peptide-bound Hyp (total Hyp) in urine. The method combines the performance simplicity and reliability of HPLC with the specificity and sensitivity of NBD-Cl pre-column derivatization of imino acids and fluorometric detection of the derivatives.

EXPERIMENTAL

Materials

Materials and their sources were as follows: 4-hydroxy-L-proline, L-proline, 3,4-dehydro-L-proline, NBD-Cl and OPA from Sigma (St. Louis, MO, U.S.A.); reagent-grade sodium dihydrogen phosphate, trichloroacetic acid (TCA) and boric acid from Carlo Erba (Milan, Italy); methanol and acetonitrile (HPLC grade) from Violet (Rome, Italy). Water was demineralized and glass-distilled. Sep-Pak C_{18} cartridges were purchased from Waters Assoc. (Milford, MA, U.S.A.) and were attached to Eppendorf combitips (Eppendorf, Hamburg, F.R.G.) to facilitate sample application and elution.

Biological samples

Blood and urine samples were obtained from healthy volunteers. During three days before sample collection, the donors were subjected to a Hyp-free diet. Urines (24-h) were combined. Blood plasma and urine samples were frozen at -20°C if not immediately processed.

NBD-Cl and OPA reagents

A 25 m*M* NBD-Cl solution was prepared in methanol. OPA reagents were prepared at 150 m*M* concentration in 0.2 *M* borate buffer, pH 9.0 (pH was adjusted with 2 *M* potassium hydroxide), or in methanol.

Standards

Two stock solutions of the analytes in 0.01 *M* hydrochloric acid were prepared. The first contained Hyp and Pro at 2 m*M* and 20 m*M*, respectively. The second contained Hyp at 20 m*M*. A stock solution of the internal standard was obtained by dissolving 3,4-dehydro-L-proline in 0.01 *M* hydrochloric acid up to 4 m*M*. These solutions were stored in a refrigerator (about 4°C) and freshly prepared every two weeks.

Analytical procedure

Plasma samples. A 0.75-ml sample of plasma was pipetted into a plastic 5-ml centrifuge tube and spiked with 200 nmol of 3,4-dehydro-L-proline (50 μl of the internal standard solution). Next, 0.2 ml of 20% (w/v) cold TCA solution was added and the tube was vortexed for 30 sec. After standing on ice for 10 min, the mixture was centrifuged at 15 000 *g* for 10 min at 4°C. The supernatant was decanted and immediately analysed or stored at −20°C for analysis within three days. A 0.1-ml aliquot of the plasma extract was added to a glass tube with a screw cap containing 0.1 ml of 0.4 *M* potassium borate buffer, pH 9.0, and 50 μl of OPA reagent in methanol. After 3 min standing at room temperature, 0.1 ml of NBD-Cl reagent was added to the mixture and the derivatization was carried out at 60°C for 3 min in the dark. The reaction was quenched by the addition of 0.65 ml of cold mobile phase; 50 μl of the solution were injected into the chromatograph.

Urine samples. A urine sample (e.g. 5 ml) was adjusted to pH 4.7—4.8 with 2 *M* hydrochloric acid. The sample was heated in a boiling water bath for 5 min and the precipitated protein removed by centrifugation at 10 000 *g* for 10 min at 4°C. A 0.5-ml aliquot of the supernatant was hydrolysed under vacuum in 6 *M* hydrochloric acid at 105°C for 16 h in a sealed ampoule. The hydrolysate was spiked with 300 nmol of internal standard, diluted 1:4 with water and evaporated to dryness at 40°C on a rotavapor. The residue was dissolved in 5 ml of 0.2 *M* potassium borate buffer, pH 9.0. A 1-ml aliquot of the sample was mixed with 0.5 ml of the OPA reagent in borate buffer. After 3 min at room temperature, 1 ml of the derivatization mixture was passed through a Sep—Pak C_{18} cartridge previously conditioned with 10 ml of methanol and 10 ml of distilled water. The first 0.5 ml of eluate was discarded and the non-derivatized imino acids were eluted with 0.2 *M* potassium borate buffer, pH 9.0, containing 10% methanol, and 2 ml were collected. A 0.2-ml sample of the eluate was added to a screw-capped glass tube containing 0.1 ml of NBD-Cl reagent and 50 μl of methanol. The derivatization was carried out at 60°C for 3 min in the dark and the reaction stopped by adding to the mixture 1.65 ml of cold mobile phase. A 50-μl aliquot of the solution was injected into the column.

Reversed-phase chromatography

The apparatus used consisted of a Violet Model Clar 002 constant-flow pump and of a Shimadzu FC 530 fluorescence spectromonitor (Shimadzu, Kyoto, Japan), equipped with a xenon lamp and a 12-μl quartz flow cell. The NBD derivatives were detected by setting the monochromators at 470 nm for excitation and 530 nm for emission. The detector was connected to a Model 7123A strip chart recorder (Hewlett-Packard, San Diego, CA, U.S.A.). A

Rheodyne 7125 valve-loop injector (Cotati, CA, U.S.A.), fitted with a 100-μl loop, was employed and the separation was performed on a 15 cm × 4 mm I.D. Spherisorb ODS 2, 5 μm particle size, column. A Guard-Pak pre-column module (Waters Assoc.) fitted with a C_{18} cartridge was used as a pre-column. The separation was carried out isocratically, by using a mixture of acetonitrile—0.1 *M* sodium phosphate buffer, pH 7.2 (9:91, v/v) as mobile phase, delivered at 1.3 ml/min at room temperature. Prior to its mixing with acetonitrile, the buffer was filtered through a 0.45-μm Millipore filter and the mobile phase was degassed by ultrasonication prior to use.

Quantitation

The concentrations of the analytes were determined on the basis of their respective calibration curves and quantitation was aided by the addition of internal standard to the samples.

To construct the plasma calibration curve, working standard solutions of 5, 10, 25 and 50 μM Hyp and 50, 100, 250 and 500 μM Pro were prepared by diluting the stock solution of the analytes with a 4% TCA solution. To each working solution was added 3,4-dehydro-L-proline up to a final concentration of 200 μM. Aliquots (0.1 ml) of the solutions were treated according to the derivatization procedure used for the plasma extract.

To construct the urine calibration curve, the stock solution of Hyp was diluted to 50, 100, 250 and 500 μM with 6 *M* hydrochloric acid. To each solution internal standard at 600 μM concentration was added. Aliquots (0.5 ml) were diluted 1:4 with distilled water and evaporated to dryness at 40°C on a rotavapor. The residue was dissolved in 5 ml of 0.2 *M* potassium borate buffer, pH 9.0, and 1 ml of each reconstituted standard solution was processed as the hydrolysed urine sample.

A 50-μl aliquot of each working standard was injected into the column. Attenuation detector sensitivity was set at × 1, except in the plasma calibration analyses when it was turned to × 4 5 min after the injection. To construct the calibration curves, peak heights of derivatized standard/derivatized internal standard versus concentration of standards were plotted. The standard curves were analysed by linear regression analysis to determine linearity.

Precision

To evaluate the precision of the method, within-run and between-run coefficients of variation (C.V.) were calculated from the same sample of plasma acid extract or urine hydrolysate. To determine within-run C.V., the analyte concentrations were calculated from five assays using the corresponding standard curves. To calculate the between-run C.V., five 0.12-ml aliquots of a TCA plasma extract and five 1.2-ml aliquots of a hydrolysed urine sample were frozen for analysis on five subsequent days. Standard curves were constructed for each everyday run. Concentrations were determined in duplicate and mean values of the five runs were used to calculate the between-run C.V.

RESULTS AND DISCUSSION

Chromatographic system

The chromatographic profile shown in Fig. 1A demonstrates a typical

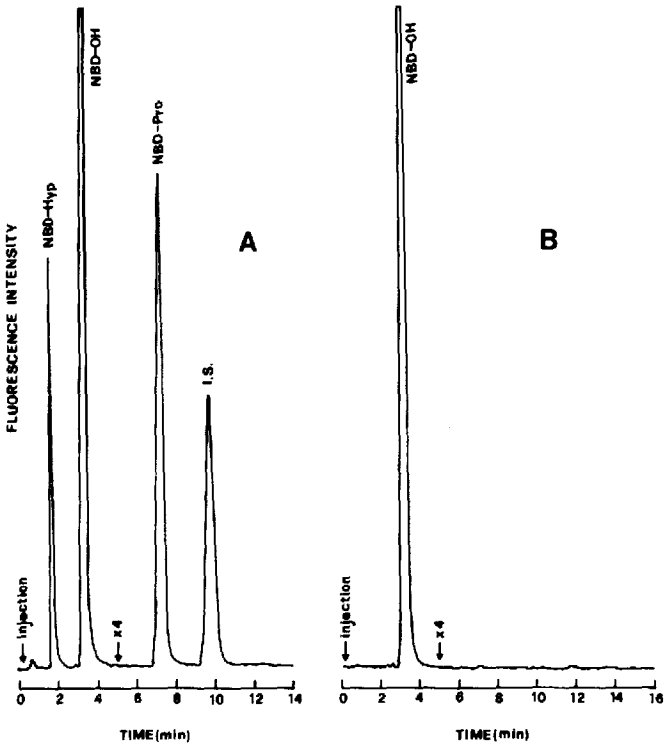

Fig. 1. (A) Chromatogram of standard Hyp, Pro and internal standard (I.S.) derivatized according to the plasma calibration procedure. Analyte peaks correspond to injected amounts of 50 pmol of Hyp and 500 pmol of both Pro and internal standard. (B) Chromatogram of a reagent blank. Chromatographic conditions: 15 cm × 4 mm I.D. column of Spherisorb ODS 2, particle size 5 μm; mobile phase, acetonitrile—0.1 *M* sodium phosphate buffer, pH 7.2, (9:91, v/v); flow-rate, 1.3 ml/min; fluorometer wavelengths, 470/530 nm; 5 min after injection, attenuation detector sensitivity is turned from × 1 to × 4.

separation of the NBD derivatives of Hyp, Pro and internal standard. The retention times of the analytes, eluted as separate symmetrical peaks, were 1.8 min for Hyp, 7.2 min for Pro and 9.6 min for internal standard. Fig. 1B shows that no interfering peaks occurred at these retention times when a reagent blank sample was chromatographed; also it can be seen that, with the derivatization procedure employed, a single not interfering peak due to the side-product 7-nitro-4-benzofurazanol (NBD-OH) appears in the chromatogram. Moreover, in a sample of standard derivatized with NBD-Cl in the absence of OPA, the presence of OPA in the injected sample does not modify the fluorometric detection (not shown).

The mobile phase was chosen after several trials performed at different buffer pH values and different organic modifier concentrations. The optimized conditions allow an efficient separation of the imino acid derivatives, with satisfactory capacity ratios, although the elution was carried out under isocratic conditions and the carboxylic group of the NBD derivatives is dissociated at the pH of the mobile phase.

The linearity of detector response to analyte derivative concentration, checked to ensure the reliability of the procedure in quantitative analysis, was

TABLE I

WITHIN-RUN AND BETWEEN-RUN REPRODUCIBILITY OF REVERSED-PHASE QUANTITATION OF HYDROXYPROLINE AND PROLINE

Figures from five analyses are given in μmol/l for plasma and in μmol/g creatinine for urine.

	Plasma		Urine
	Hyp	Pro	Hyp
Within-run			
Mean	9.2	232.0	122.0
Standard deviation	0.2	6.5	5.2
Coefficient of variation (%)	2.2	2.8	4.2
Between-run			
Mean	10.4	202.7	185.8
Standard deviation	0.4	8.3	9.1
Coefficient of variation (%)	3.8	4.1	4.9

excellent for both Hyp and Pro. Plasma calibration curves of peak ratios against Hyp and Pro concentration showed linearity over ten times the concentration range examined. Correlation coefficients of 0.999 for Hyp and Pro were obtained from linear regression analysis. The corresponding regression equations were: $y = 0.14x + 0.09$ for Hyp and $y = 0.0093x + 0.18$ for Pro. Linear regression analysis of the urine calibration curve for Hyp showed a correlation coefficient of 0.999 and a regression equation equal to $y = 0.0042x - 0.020$.

Precision analysis of Hyp and Pro in five identical aliquots of the same plasma extract and of Hyp in five specimens of the same urine are summarized in Table I. The within-run coefficients of variation for the quantitation of Hyp (2.2%) and Pro (2.8%) in plasma and of Hyp (4.2%) in urine indicate a good reproducibility of the analysis. The between-run coefficients of variation were found to be not higher than 4.9% both for plasma and urine analyses. The detection limits were estimated to be 1 and 5 injected pmol of Hyp and Pro, respectively.

Chromatography of biological samples

HPLC methods using pre-column NBD-Cl derivatization have previously been applied to the determination of Hyp and Pro in standard solutions [11] and of Hyp in purified collagen samples [9]. However, several analytical difficulties arise in the determination of Hyp in biological fluids such as plasma (serum) or urine. The drawbacks occur either because of the very low concentration of the analyte or because of the complexity of the sample matrix. HPLC of a plasma acid extract, derivatized with NBD-Cl without previous reaction with OPA, does not allow Hyp to be quantified because of the presence of several interfering compounds, mostly amino acids, in the chromatographic profile. Moreover, the analytical difficulties related to the matrix complexity are particularly increased when urine samples are analysed. For these reasons the procedure for Hyp (and Pro) determination using pre-column derivatization greatly depends upon the sample matrix, which determines the degree of sample clean-up required prior to the analytical chromatography. Thus, a

satisfactory clean-up of interfering amino acids in plasma samples is obtained by preliminary derivatization with OPA. This treatment of the plasma sample allows the selective derivatization of imino acids with NBD-Cl and the immediate determination of derivatives by HPLC. A further clean-up step of the OPA-derivatized sample on a Sep-Pak C_{18} cartridge is, however, required for total Hyp determination in urine.

Fig. 2 shows the chromatographic profiles of derivatized samples of plasma and urine, processsed as described above. Solute peaks were identified by their capacity ratios from comparison with derivatized reference compounds and by addition of known amounts of standards to the biological samples. Moreover, in order to unequivocally identify the NBD-Hyp peak and to obtain evidence that no other interfering compounds were present, this chromatographic peak from analysis of a 0.1-ml derivatized urine sample was collected and diluted 1:5 with distilled water. The material was adsorbed on a Sep-Pak C_{18} cartridge activated with methanol and washed with distilled water. After washing with 5 ml of distilled water, the material was eluted with 5 ml of methanol. The fraction was dried, resuspended in a minimal volume of methanol and subjected

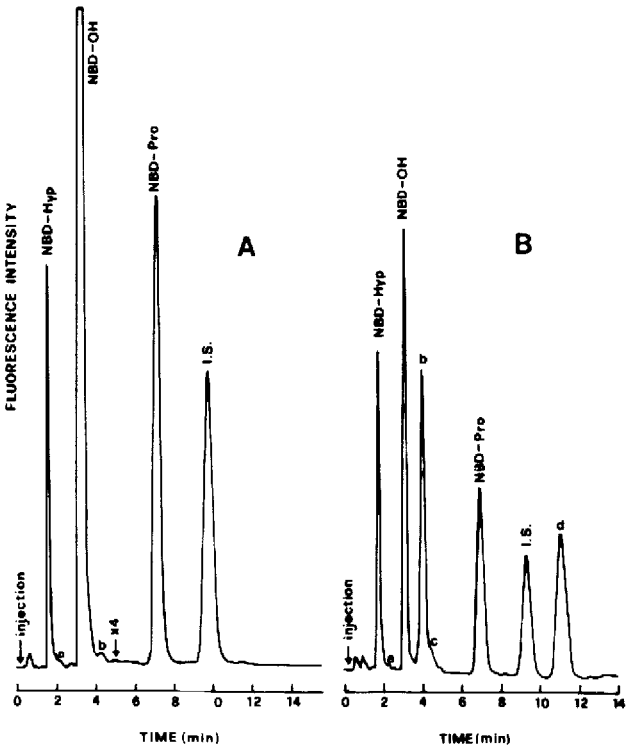

Fig. 2. (A) Representative chromatogram of free Hyp and Pro in a plasma sample, derivatized as described in the text. The calculated concentrations for Hyp and Pro are 13.0 and 138.0 μmol/l, respectively. Chromatographic conditions are as described in Fig. 1. Peaks a and b are unknown. (B) Typical chromatogram of total Hyp in urine. The calculated concentration for Hyp is 152.0 μmol/g creatinine. Chromatographic conditions are as described in Fig. 1, except that attenuation detector sensitivity is maintained at $\times 1$. Peaks a, b, c, and d are unknown.

to thin-layer chromatography (TLC) as described by Bellon et al. [12]. Only one fluorescent spot, with the same R_F as authentic NBD-Hyp, was observed.

The procedure described here was used to assess the concentration range of Hyp and Pro in plasma and of total Hyp in urine from 21 healthy adult volunteers. The amounts of free Hyp and Pro in plasma determined by the present method were 8.5 ± 0.8 μmol/l and 251 ± 36 μmol/l, respectively. These figures agree with those obtained by ion-exchange chromatography using the post-labelling method employing NBD-Cl [7] and OPA—hypochlorite [5]. The level of total Hyp in urine was 158 ± 35 μmol/g creatinine. Similar results were obtained by the fluorometric TLC determination [12], which requires some time-consuming clean-up steps of the urine sample prior to TLC determination. On the other hand, higher figures were obtained by the conventional colorimetric assay with *p*-dimethylaminobenzaldehyde [13, 14].

Finally, due to its accuracy and precision, in addition to its ease of performance, the method may be regarded as a useful biomedical application of liquid chromatography to the determination of Hyp and Pro in both plasma and urine.

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