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Short Communication

Determination of proline by reversed-phase high-performance liquid chromatography with automated pre-column *o*-phthaldialdehyde derivatization

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ABSTRACT

A simple and sensitive fluorometric HPLC method was developed for the analysis of proline in biological samples. The HPLC apparatus consisted of an autosampler, a binary solvent delivery system, a 3-μm reversed-phase C_{18} column (150 × 4.6 mm I.D.) guarded by a 40-μm reversed-phase C_{18} column (50 × 4.6 mm I.D.), a fluorescence detector, and a computer workstation. Proline was oxidized to 4-amino-1-butanol in the presence of chloramine-T and $NaBH_4$ in a 60°C water bath, which took 11 min. 4-Amino-1-butanol was automatically derivatized with *o*-phthaldialdehyde in the presence of 2-mercaptoethanol. The proline derivative was separated on a 25-min gradient program, employing solvent A (0.1 *M* sodium acetate–0.5% tetrahydrofuran–9% methanol; pH 7.2) and solvent B (methanol). Fluorescence was monitored with excitation at 340 nm and emission at 450 nm. The conversion of proline to 4-amino-1-butanol was quantitative, reproducible, and linear with proline concentrations ranging from 25 to 500 μM commonly present in biological samples. The described method can be readily used for quantifying proline in biomedical research.

INTRODUCTION

A number of sensitive methods have been developed for analysis of amino acids including proline, using high-performance liquid chromatography (HPLC) during the last 10 years. These include the pre-column derivatization of amino acids with 4-chloro-7-nitrobenzofurazan (NBD-Cl) [1], 9-fluorenyl methylchloroformate (FMOC) [2,3], and phenylisothiocyanate (PITC) [4]. The NBD-Cl method not only requires the heating of both the reactor and the reaction coil at 65°C but also gives relatively low detection sensitivity for proline derivative and considerable side reactions [1]. The FMOC method calls for (1) the absolute pre-column removal of the excess FMOC, which is highly fluorescent itself, either by multiple extractions with pentane [2] or by reaction with another added chemical [3], (2) additional heating facilities to maintain the analytical column at 45°C [3], and (3) the need of highly qualified detectors with liquid filters due to the fluorescent property of the FMOC derivatives [2,3]. On the other hand, the PITC method is associated with multiple steps of pre-column sample preparations, including lyophilization and evaporation of reacting solutions, amino acid derivatization, and purification of

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amino acid-derivatives by ion-exchange chromatography for biological samples. In addition, this method requires a long running time of 105 min (including 25 min for column regeneration) for separation of proline in physiological samples [4].

The analysis of primary amino acids by reversed-phase HPLC using the pre-column derivatization with *o*-phthalaldehyde (OPA) has been widely performed in many laboratories [5–8]. This is largely owing to the many advantages offered by the OPA method. These include (1) the rapid derivatization of amino acids with OPA and the efficient separation of amino acid derivatives at room temperature, (2) high sensitivity of detection (pmol range), (3) easy automation on the HPLC apparatus, and (4) few, if any, interfering side reactions. However, there is a significant problem with this OPA method in that proline does not react directly with OPA [5–8]. Attempts to overcome this shortcoming have involved the post-column [9,10] and pre-column [1,11] oxidation of the imino acid ring of proline, using chlorinated oxidizing agents.

It is noteworthy that Cooper *et al.* [12] introduced a simple procedure for the oxidation of the imino acid ring of proline using chloramine-T in the presence of borohydride, which probably yielded 4-amino-1-butanol. The study of Cooper *et al.* [12], however, involved only serine and imino acid standards. It is not known if there are any interfering reactions between the derivatizing agents (chloramine-T and $NaBH_4$) and the amino acids or other compounds found in physiological samples. As a result, the general usefulness of the Cooper *et al.*'s oxidation method for proline analysis in biomedical research remains to be determined. The objective of this study was therefore to investigate the potential use of the Cooper *et al.*'s method [12] in quantifying proline in biological samples.

MATERIALS AND METHODS

Chemicals

All L-amino acids, sodium borate, lithium hydroxide, chloramine-T, dimethyl sulfoxide, sodium borohydride, benzoic acid, sodium acetate, *o*-phthaldialdehyde, tetrahydrofuran, 2-mercaptoethanol, and Brij-35 were purchased from Sigma (St. Louis, MO, USA). 4-Amino-1-butanol was obtained from Aldrich (Milwaukee, WI, USA). HPLC-grade methanol and HPLC-grade water were purchased from Fisher Scientific (Houston, TX, USA) and were used throughout the study.

Plasma sample

Jugular vein blood (5 ml) was withdrawn from 28-day-old pigs into heparinized tubes and immediately centrifuged at 1000 *g* for 10 min. The plasma (supernatant) was then collected. An aliquot of 1 ml of plasma was deproteinized with 1 ml of 1.5 *M* $HClO_4$. The supernatant was neutralized with 0.5 ml of 2 *M* K_2CO_3 and used for the analysis of free proline.

Milk sample

An aliquot of 10 ml of milk was manually collected from sows on day 28 of lactation. The milk was immediately centrifuged at 1000 *g* for 10 min to remove fat and suspended cells including lymphocytes and macrophages. Defatted milk (1 ml) was deproteinized with 1 ml of 1.5 *M* $HClO_4$. The supernatant was neutralized with 0.5 ml of 2 *M* K_2CO_3 and used for the analysis of free proline. The precipitated milk protein was washed 3 times with 10 ml of deionized water, air-dried, and then suspended in 5 ml of 6 *M* HCl for hydrolysis at 110°C for 24 h under nitrogen. The hydrolysates were evaporated by freeze-drying, and the residues were dissolved in 10 ml of HPLC-grade water for the analysis of protein-bound proline.

Skeletal muscle

Gastrocnemius muscles were isolated from 3-month old rats. Muscle (1 g) was homogenized in 1 ml of 1.5 *M* $HClO_4$, and the homogenates were centrifuged at 1000 *g* for 10 min. The supernatant was neutralized with 0.5 ml of 2 *M* K_2CO_3, and used for the analysis of free proline.

Incubated enterocytes

Enterocytes (absorptive cells in the small intestine) were prepared from 29-day old pigs as described by Watford *et al.* [13]. Cells (10 mg protein) were incubated at 37°C for 30 min in 2

ml of oxygenated (95% O_2/5% CO_2) Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 5 m*M* L-glutamine. The incubation was terminated by addition of 0.2 ml of 1.5 *M* $HClO_4$. The acidified medium was neutralized with 0.1 ml of 2 *M* K_2CO_3, and the supernatant was used for proline analysis.

Recovery of proline from plasma, milk, protein hydrolysates, muscle extracts, and enterocyte extracts

The recovery rates of proline from plasma, milk, protein hydrolysates, muscle extracts, and enterocyte extracts were checked by adding 60 μl of 0.5 m*M* proline or water to 0.2 ml of plasma, milk, protein hydrolysates, muscle extracts, or enterocyte extracts. The processing of the samples for proline analysis was performed as described above.

Conversion of proline to 4-amino-1-butanol

The optimal conditions reported by Cooper *et al.* [12] for converting proline to probably 4-amino-1-butanol were confirmed and employed in this study. Briefly, 200 μl of 0–500 μM proline standard (amino acid standard mixture or samples) were added to 200 μl of 13.3 m*M* chloramine-T preheated at 60°C for 2 min in a water bath. Following 1 min incubation at 60°C, 200 μl of 333 m*M* $NaBH_4$ was added to the solution and the mixture was further incubated at 60°C for 10 min. After cooling at room temperature, 0.1 ml of the reaction mixture (or 0.5 ml for enterocyte samples) was directly used for proline analysis without any extraction or purification.

OPA reagent

OPA reagent was prepared by dissolving 50 mg OPA in 1.25 ml methanol, followed by addition of 11.2 ml of 0.04 *M* sodium borate (pH 9.5), 50 μl 2-mercaptoethanol, and 0.4 ml Brij-35 [1]. Although the prepared OPA reagent is usually stable for more than 24 h, it is routinely

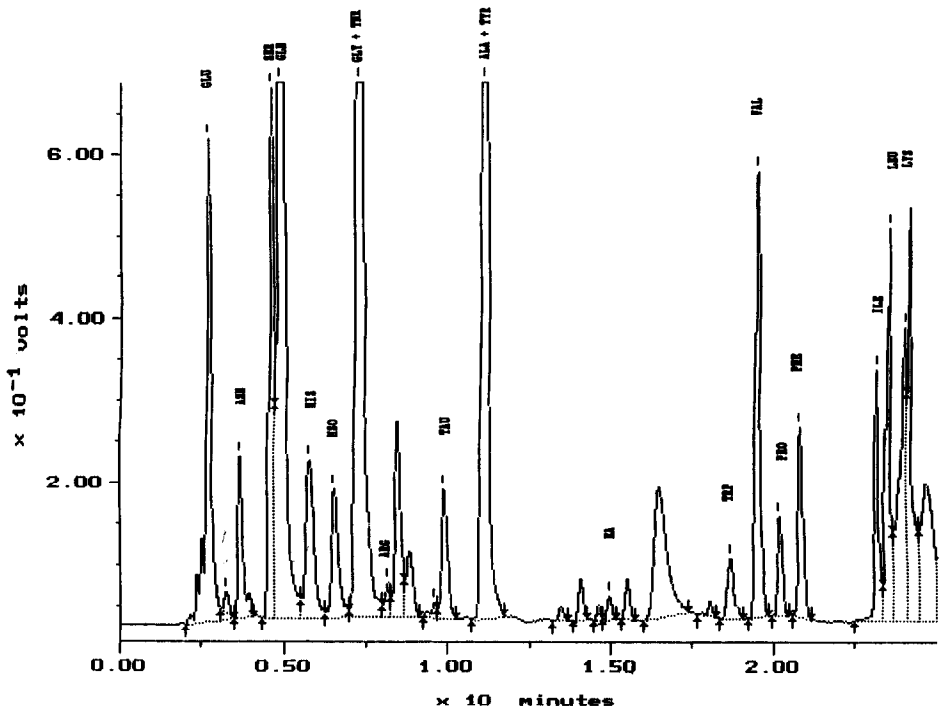

Fig. 1. Elution profiles of OPA derivatives of plasma free amino acids. An aliquot (0.2 ml) of deproteinized pig plasma was used for the conversion of proline to 4-amino-1-butanol in the presence of chloramine-T and $NaBH_4$. A portion (0.1 ml) of the reaction mixture was used for HPLC analysis as described in the text. Methionine was oxidized by chloramine-T and $NaBH_4$ to form methionine sulfoxide. Non-standard abbreviations used: MSO = methionine sulfoxide; TAU = taurine; EA = ethanolamine.

used within 24 h in the author's laboratory to cautiously ensure that all the chemicals used were of the highest quality.

HPLC apparatus and gradient

The Waters HPLC apparatus consisted of a Model 600E Powerline multisolvent delivery system with 100-μl heads, a Model 712 WISP autosampler, a Supelco 3-μm reversed-phase C_{18} column (150 × 4.6 mm I.D.) guarded by a Supelco 40-μm reversed-phase C_{18} column (50 × 4.6 mm I.D.), a Model 420-AC fluorescence detector, and a Model 810 Baseline Workstation (Waters, Milford, MA, USA). The mobile phase consisted of solvent A (0.1 *M* sodium acetate–0.5% tetrahydrofuran–9% methanol; pH 7.2) and solvent B (methanol), with a combined total flow-rate of 1.1 ml/min. Solvent A was prepared by dissolving 27.3 g sodium acetate (trihydrate) in 1.6 l water, followed sequentially by adjusting solution pH to 7.2 with 6 *M* HCl, adding 10 ml tetrahydrofuran and 180 ml methanol, and adjusting the solution to a final volume of 2 l with water. A gradient program with a total running time of 25 min (including the time for column regeneration) was developed for satisfactory separation of proline (0 min, 30% B; 10 min, 45% B; 17 min, 55% B; 17.1 min, 100% B; 19 min, 100% B; 19.1 min, 30% B; 24.5 min, 30% B).

Chromatographic procedure

All chromatographic procedures were performed at room temperature. An aliquot (0.1 ml) of amino acid standards or derivatized samples (or 0.5 ml for enterocyte samples) was mixed with 0.1 ml of 1.2% benzoic acid (to prevent possible growth of bacteria) and 1.5 ml water in a 5-ml glass vial. A brown vial containing 4 ml OPA reagent was placed onto the Waters 712 WISP autosampler, which was programmed to inject 25 μl sample and 25 μl OPA

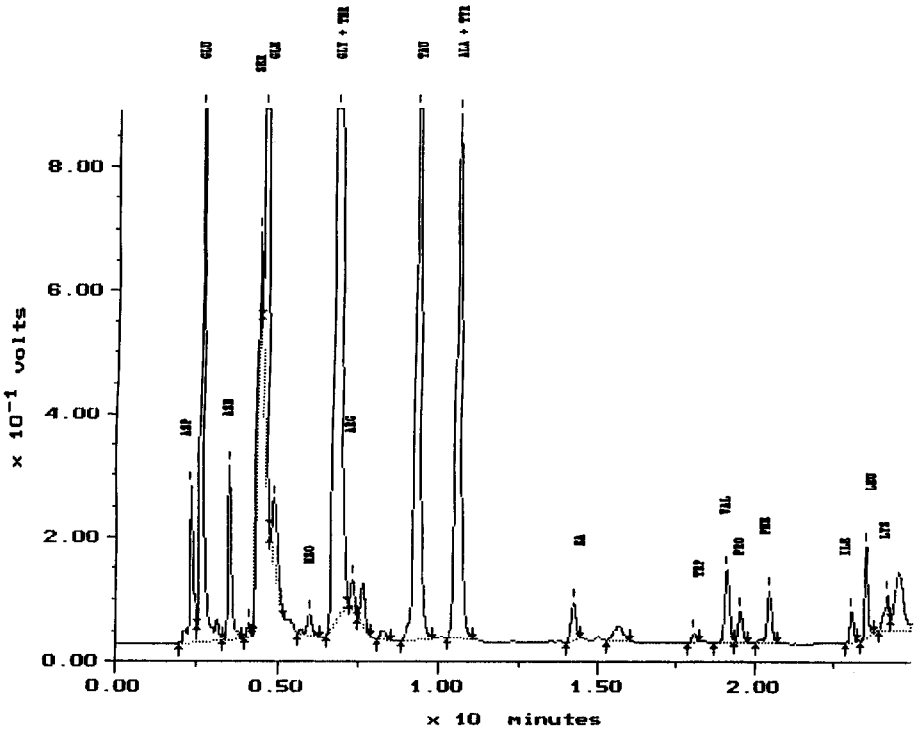

Fig. 2. Elution profiles of OPA derivatives of milk free amino acids. An aliquot (0.2 ml) of the supernatant from deproteinized milk was used for the conversion of proline to 4-amino-1-butanol in the presence of chloramine-T and $NaBH_4$. A portion (0.1 ml) of the reaction mixture was used for HPLC analysis as described in the text. Non-standard abbreviations used: MSO = methionine sulfoxide; TAU = taurine; EA = ethanolamine.

reagent into the HPLC column without a delay mixing time. Fluorescence was monitored with excitation at 340 nm and emission at 450 nm. Amino acids and proline-derived 4-amino-1-butanol were identified with the aid of authentic standards. Peak integrations were performed by a Waters Model 810 Baseline Workstation.

RESULTS AND DISCUSSION

4-Amino-1-butanol was previously proposed to be the oxidation product of proline in the presence of chloramine-T and $NaBH_4$ [12]. This suggestion was supported by the following lines of evidence from the present study. First, proline standard treated with chloramine-T and $NaBH_4$ yielded a peak which was co-eluted with authentic 4-amino-1-butanol standard (data not shown). There were no side reactions or interfering peaks on the resulting HPLC chromatograms. Second, there was no peak for 4-amino-1-butanol on HPLC chromatograms, when a mixture of all amino acid standards but proline were treated with chloramine-T and $NaBH_4$. This indicates that amino acids other than proline were not converted to 4-amino-1-butanol under the conditions used. Third, when a mixture of all amino acid standards containing proline (without 4-amino-1-butanol) were treated with chloramine-T and $NaBH_4$, there was a peak with the same retention time as 4-amino-1-butanol, which was satisfactorily separated from all other amino acid derivatives (data not shown).

The linearity of the oxidation of proline to 4-amino-1-butanol with proline concentrations from 25 to 500 μM was obtained ($r = 0.996$, $n = 7$, $P < 0.01$). The conversion of proline to 4-amino-1-butanol was quantitative, reproducible, and linear with proline concentrations ranging from 25 to 500 μM which are commonly present in biological samples. The standard errors were less than 3% of the means at each of the proline concentrations used. This indicates the good reproducibility of both the proline

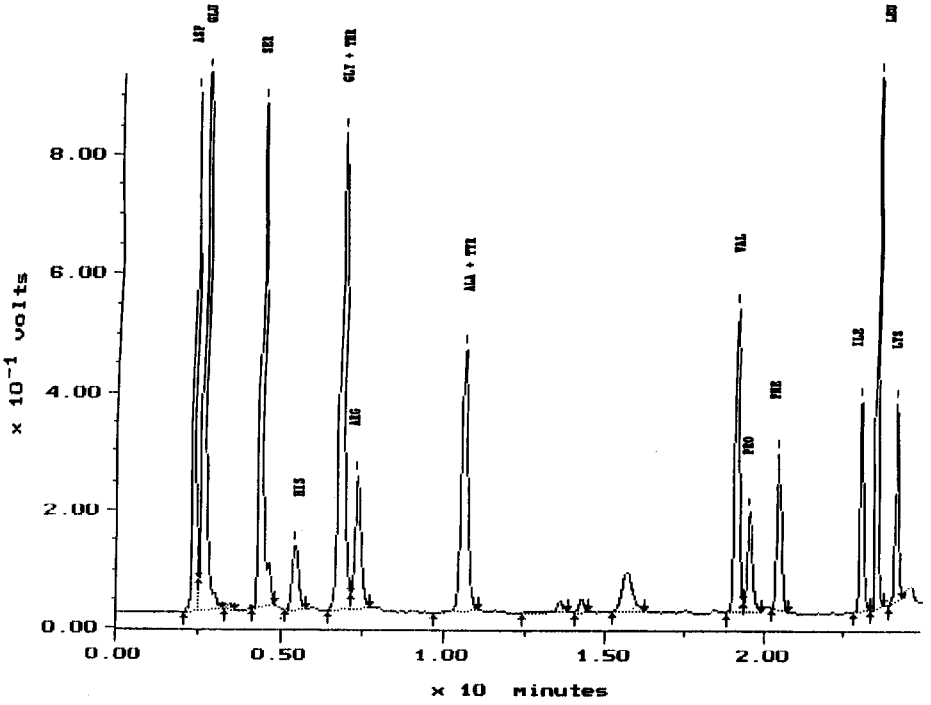

Fig. 3. Elution profiles of OPA derivatives of milk protein-bound amino acids. An aliquot (0.2 ml) of milk protein hydrolysates was used for the conversion of proline to 4-amino-1-butanol in the presence of chloramine-T and $NaBH_4$. A portion (0.1 ml) of the reaction mixture was used for HPLC analysis as described in the text.

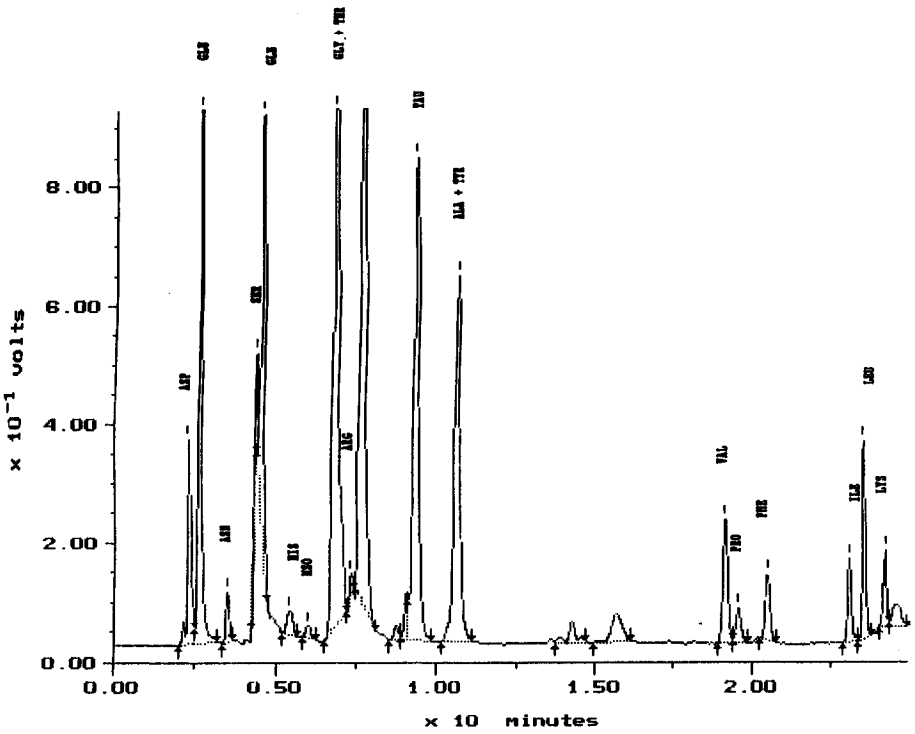

Fig. 4. Elution profiles of OPA derivatives of gastrocnemius muscle free amino acids. An aliquot (0.2 ml) of muscle extracts was used for the conversion of proline to 4-amino-1-butanol in the presence of chloramine-T and $NaBH_4$. A portion (0.1 ml) of the reaction mixture was used for HPLC analysis as described in the text. Non-standard abbreviations used: MSO = methionine sulfoxide; TAU = taurine.

derivatization procedure and the HPLC analysis. The detection limit for the proline derivative, 4-amino-1-butanol, was 2.5 pmol, when using the Waters 420-AC Fluorescence Detector at a gain setting of 8 (maximum setting of up to 128). This high detection sensitivity is sufficient enough for the analysis of proline in various physiological samples.

The fluorometric HPLC method as described above was satisfactorily applied to the analysis of proline in biological samples, including plasma (Fig. 1), milk (Fig. 2), milk protein (Fig. 3), skeletal muscle (Fig. 4), and enterocyte extracts (Fig. 5). The satisfactory resolution of proline-derived 4-amino-1-butanol from other amino acid derivatives were achieved, using a 25-min gradient program (including the time for column regeneration). It is evident from Figs. 1–5 that proline was readily detected in all the samples used, even though they were diluted by 50 times (or 10 times for enterocyte extracts) before use for HPLC analysis (see the Materials and methods section). It is noteworthy that there were minimal interfering peaks on the HPLC chromatograms for all the samples studied (Figs. 1–5), demonstrating the absence of side reactions in the oxidation of proline to 4-amino-1-butanol. These results indicate that the pre-column oxidation of proline to 4-amino-1-butanol with chloramine-T and $NaBH_4$ [12] is suitable for the analysis of proline in biological samples including incubated cells. This sensitive HPLC method for proline analysis is useful for quantifying proline concentrations in both cellular fluids and tissue proteins and for studying its metabolism in animal cells. For example, the low concentration of proline in the extracts of the pig enterocytes incubated with 5 m*M* glutamine, relative to glutamate and aspartate (Fig. 5), suggests that proline is not a major product of glutamine metabolism in these cells. The present simple method was also readily

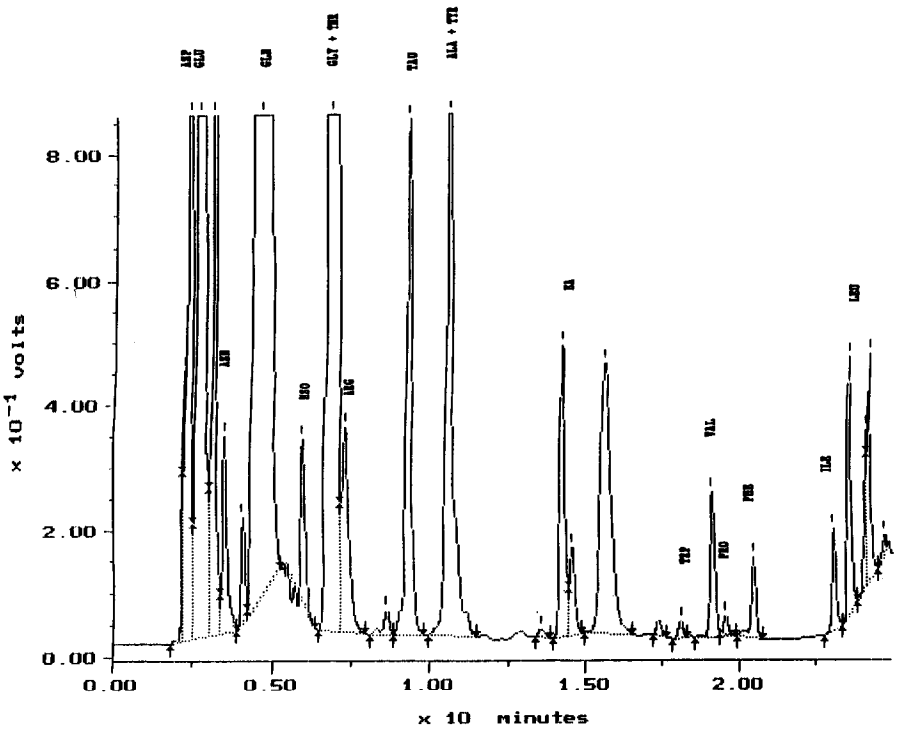

Fig. 5. Elution profiles of OPA derivatives of free amino acids in pig enterocyte extracts. An aliquot (0.2 ml) of enterocyte extracts was used for the conversion of proline to 4-amino-1-butanol in the presence of chloramine-T and $NaBH_4$. A portion (0.5 ml) of the reaction mixture was used for HPLC analysis as described in the text. Non-standard abbreviations used: MSO = methionine sulfoxide; TAU = taurine; EA = ethanolamine.

employed to determine free proline in rat livers and kidneys (data not shown).

The recovery rates (means ± S.D., $n = 6$) of proline from plasma, milk, protein hydrolysates, skeletal muscle extracts, and enterocyte extracts were 90.6 ± 2.1, 85.3 ± 2.6, 96.3 ± 2.7, 88.7 ± 2.4, and 97.8 ± 3.1%, respectively. The coefficients of variation ranged from 2.3% for the plasma to 3.1% for the enterocyte extracts. This further demonstrates the good reliability of the present method for proline analysis in biological samples.

The present procedure for proline analysis in the biological samples offers some advantages. First, there is no need for any pre-column purification of amino acid derivatives in contrast to the PITC method [4], or for the removal of excess reagents prior to HPLC analysis as absolutely required by the FMOC method [2,3]. Second, both rapid derivatization of 4-amino-1-butanol with the OPA reagent and efficient separation of 4-amino-1-butanol from other amino acid derivatives are achieved at room temperature. This facilitates an easy automation procedure on the HPLC apparatus and does not require additional facilities on the HPLC apparatus to maintain the column or the reactor and the reaction coil at high temperatures as rigidly needed in the FMOC (45°C) [3] and NBD-Cl (65°C) [1] methods, respectively.

In conclusion, proline was quantitatively oxidized to 4-amino-1-butanol in the presence of chloramine-T and $NaBH_4$ in a 60°C water bath, which took 11 min. 4-Amino-1-butanol was automatically derivatized with OPA and satisfactorily separated from other amino acid derivatives on a 25-min gradient program, using reversed-phase HPLC. This fluorescent method is sensitive and reliable, and can be readily used for quantifying proline in biological samples.

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