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EFFECT OF pH ON SPECTRAL CHARACTERISTICS OF P5C-NINHYDRIN DERIVATIVE: APPLICATION IN THE ASSAY OF ORNITHINE AMINO TRANSFERASE ACTIVITY FROM TISSUE LYSATE

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ABSTRACT

Currently available method(s) for assaying pyrroline-5-carboxylate (P5C), an important intermediate metabolite of ornithine, proline and glutamate metabolic pathways, are cumbersome or not sensitive enough for microanalysis. The present study involving the synthesis of P5C followed by purity check, molecular mass (amu =113.1) determination by mass spectrometry and spectral characterization of P5C-ninhydrin derivative (λ max: 510 nm) confirmed the authenticity of the preparation. Studies on the effect of pH on spectral characteristics of P5C ninhydrin derivative demonstrated a significant change with respect to λ max (620 nm) and several ~ 12 fold increase in molar extinction coefficient (ε: 1.96 x 10^5) in alkaline conditions (pH:7.0-8.0) as compared to the reported Molar ε of 1.65 x 10^4 at λ max 510 nm in ethanolic solution. The modified method, with the improved sensitivity, is adopted for the assay of ornithine amino transferase activity in WBC's/platelets lysate(s) from human blood.

KEY WORDS

Pyrroline-5-Carboxylate, Mass spectrometry, WBC's/platelets lysate(s), Ornithine amino-transferase, Pyrroline-5-Carboxylate-Ninhydrin complex.

INTRODUCTION

Pyrroline-5-Carboxylate (P5C), an obligatory intermediate metabolite of proline, ornithine and glutamate with a double bond in the Δ^1 position of the five carbon ring links tricarboxylic acid and the urea cycle(s) (1). Metabolically, the tissue level of P5C is regulated by ornithine amino transferase (EC: 2.6.1.13), proline oxidase (No EC number assigned) / P5C-reductase (EC: 1.5.1.2) and P5C-synthase (No EC number assigned) / P5C-dehydrogenase (EC: 1.5.1.12) (2). P5C and proline, constituting a redox pair, utilizing pyridine nucleotides (NADP/NADPH) as the cofactor (3) has an important role in redox- dependent metabolic pathways (4,5). Compartmentalization of enzymes involved in the inter conversion of ornithine, P5C and proline across the sub cellular compartments of mitochondria and cytoplasm respectively (6) and the tissues such as hepatocytes and erythrocytes (2) suggest that P5C could be a shuttling proton acceptor with a physiological role in regulating the intra and extra cellular redox status (7,8). Further, P5C as an intermediate metabolite of proline metabolism is believed to play a role in cell death in animals and plants (9). In spite of the importance of P5C in overall intermediate metabolism, little is known about the circulatory / tissue level and urinary excretion of P5C. The lacunae in our understanding of the role of P5C in health and diseases appears to due to the methodological constrains, in terms of insensitivity of the currently available assay methods and possible labile nature of the compound (P5C) of interest. Nevertheless, methods based on o-aminobenzaldehyde (10) and ninhydrin (11) derivatisation of P5C has been successfully used to monitor the formation of the product (P5C) of OAT activity in tissues.

The present study deals with the chemical synthesis and

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characterization of pyrroline -5 -carboxylate (P5C) and the effect of pH on spectral characteristics of the P5C-Ninhydrin derivative with an objective of improving its analytical sensitivity. Further, the method was successfully adopted for the assay of OAT activity in WBC's/platelets lysate(s) from human blood.

MATERIALS AND METHODS

Preparation of Pyrroline-5-Carboxylate : In-house synthesis of P5C, with slight modifications, was essentially based on the method described by earlier investigators (11,12). In brief, 4.4 ml of 50 mM sodium metaperiodate (~ pH: 7.0) (Merck Ltd, India) was mixed with 2.8 ml of 70 mM of DL-5-hydroxylysine (Sigma Chem. Co USA) and allowed to remain at 4º C for 8 minutes. 70 µl of 1 M glycerol was added, allowed to stand for 2 minutes to act upon the excess periodate, followed by acidification by adding 60 µl of 6 N HCl. P5C was purified by adopting ion exchange chromatography based on batch processing instead of column chromatography.

Preparation of Ion exchange [Dowex-50W-8X] : In brief, Dowex 50W-X8 ion exchanger resin (J.T. Baker chem. Co), 10 ml bed volume, was suspended in distilled water (1:3 v/v) and gently mixed (15 minutes) in a centrifuge tube (50 ml) by using a rota-shaker (Scientific Industries Inc USA) and allowed to settle. The supernatant was siphoned off. Resin was resuspended in 3 volumes of distilled water and the wash cycles were repeated thrice. Resin was suspended in 3 volumes of 5 mM HCl by gentle mixing for 15 minutes and the supernatant was discarded. The equilibration steps were repeated thrice.

Purification of P5C : P5C synthesized, as described earlier, was quantitatively transferred to a centrifuge tube containing resin (bed volume 3 ml) and allowed to bind the resin by gentle mixing for 15 minutes. Supernatant was siphoned off and the resin was subjected to wash cycle (thrice in 5 mM HCl). Bound P5C from the resin was eluted by suspending and equilibrating (10 minutes) with 1 M HCl (5 ml). The resin was subjected to repeat elution six times in 5 mls of 1 M HCl and the supernatants were stored at 4ºC for further analysis. Aliquots from each of the elutes were checked for the presence of P5C by ninhydrin derivatisation method as described (11). P5C rich fractions were pooled together and checked for purity/ homogeneity by thin layer chromatography (TLC) followed by staining with ninhydrin.

Spectral analysis and mass determination of P5C : A pooled fraction of P5C was subjected for absorption spectrum analysis against 1M HCl as blank and spectral characteristics were recorded in UV1601PC Shimadzu spectrophotometer. An aliquot of P5C synthesized was subjected to mass spectrometry using API 3000, Applied Biosystems.

Quantification of P5C : The quantification of P5C was based on ninhydrin derivatisation method (11). In brief, 0.25 ml of P5C was mixed with equal volumes of water, perchloric acid (2.4 N), aqueous solution of ninhydrin (2% w/v) and allowed to derivatize in boiling water bath for 15 minutes. Tubes were centrifuged (2500 x g / 10 minutes) and the supernatant was discarded. Insoluble reddish sediment was dissolved in 1 ml of ethanol and absorption spectrum was scanned against reagent blank and concentration was calculated based on the earlier reported mM ε of 16.5 for the P5C-ninhydrin derivative (11) at 510 nm.

Thin Layer Chromatography (TLC) : TLC separation was carried out on pre-coated cellulose plates (EMerck Cat No 1.05552) by spotting an aliquot (~5ml) P5C solution equivalent to ~ 9 nmoles along with comparable amount of ornithine, proline, glutamate and hydroxylysine. Chromatogram was developed in a solvent system comprising Butanol: Acetone: Acetic acid: Water [35:35:10:20 v/v], followed by staining with ninhydrin reagent (0.25 g percent in acetone) and heating in hot air oven (100ºC/10 minutes).

Effect of pH on absorption spectral characteristics of P5C-ninhydrin complex : Development of P5C-ninhydrin derivatisation in duplicates was carried out essentially as described earlier for the assay of P5C. While one set of P5C-ninhydrin derivatives were dissolved in 1 ml of ethanol only, the duplicate was dissolved in 1 ml of 50% (v/v) ethanol containing 0.05M buffer of varying pH (i.e., pH: 7.0, 8.0, 9.0, 10.0). Absorption spectrum of the P5C-ninhydrin derivative at different pH conditions were recorded in spectrophotometer against corresponding reagent blanks.

Assay for ornithine amino transferase (OAT: EC: 2.6.1.13) activity in WBC's lysate(s) : WBC's and platelet's pellets, separated from 8 mls of EDTA as described in house developed method (13), were suspended separately in 0.5 ml of isotonic saline and mixed with an equal volume of Triton X-100 (0.4% v/v) and allowed to lyse for 1 hr at 4 -8 ºC. Lysate(s) were subjected to centrifugation (5000 x g/15 minutes) and the supernatants used for protein estimation by Bradford's dye binding method (14) and the assay of OAT activity (11). In brief, the enzyme assay system comprised substrates (ornithine: 35 mM; α-ketoglutarate:5 mM), cofactor (pyridoxal phosphate: 0.05 mM) in 50 mM Tris HCl buffer (pH: 8.0) of a

total volume of 1 ml. Enzyme reaction was initiated by adding cell lysate(s) (≅ 100 ± 15 µg protein) to a pre incubated (10 minutes) substrate buffer cocktail and allowed to continue for 30 minutes at 37 ºC. Enzyme activity was terminated by adding 0.25 ml of 3.6 N perchloric acid followed by adding 0.25 ml of 2 g % ninhydrin in water. Tubes were transferred to boiling water bath and allowed to derivatize for 15 minutes and centrifuged (2500 x g/15 minutes). After decanting the supernatant, the reddish brown sediment was suspended in ethanol (0.5 ml) and vortexed to extract the P5C-ninhydrin chromogen (pinkish color) followed by adding of 0.5 ml of 100 mM Tris HCl buffer, pH: 8.0 which transforms the color to bluish and centrifuged (2500 x g/10 minutes). Absorbance was measured at 620 nm and the concentration of P5C was calculated based on the established Molar ε: 1.96×10^5. The enzyme activity was expressed as nmoles of product (P5C) formed/minute/mg protein under the enzyme assay conditions described.

RESULTS AND DISCUSSION

P5C, an important intermediary metabolite of ornithine, proline and glutamate (15), together with proline constituting an oxido-reductant pair has a major role in regulating the redox status between mitochondrial and cytosolic compartments (6,16) as well as between hepatocytes and erythrocytes (2). Thus, it is imperative that disturbances in the synthetic or catabolic arms of P5C metabolism are likely to cause disease status. One such metabolic disorder where P5C synthesis is severely affected is gyrate atrophy of choroid and retina (GACR) wherein OAT deficiency results in hyper ornithinemia (17,18). It is of interest to note that the observed degenerative change of retina in GACR is often related to the associated hyperornithinemia per se, but not as due to the possible depletion of P5C leading to adverse effect on retinal pigmented epithelium (RPE), the primary target tissue affected. Reasons for not studying the metabolic relevance of P5C in health and diseases in general including GACR appear to be due to the low concentration of this metabolite in tissue sources, because of possible biological half life or the labile nature of the metabolite itself and the lack of sensitive analytical methods. Nevertheless, methods based on derivatisation of P5C with o-aminobenzaldehyde (10) and ninhydrin (11) to monitor the OAT activity have been employed by the earlier investigators. Apparently these methods appear to be not sensitive for assaying the circulatory level of P5C.

The present study involving an in-house synthesis of P5C and after having established the chemical purity, based on TLC (Fig 1) and mass spectrometry (Fig 2), also evaluated the

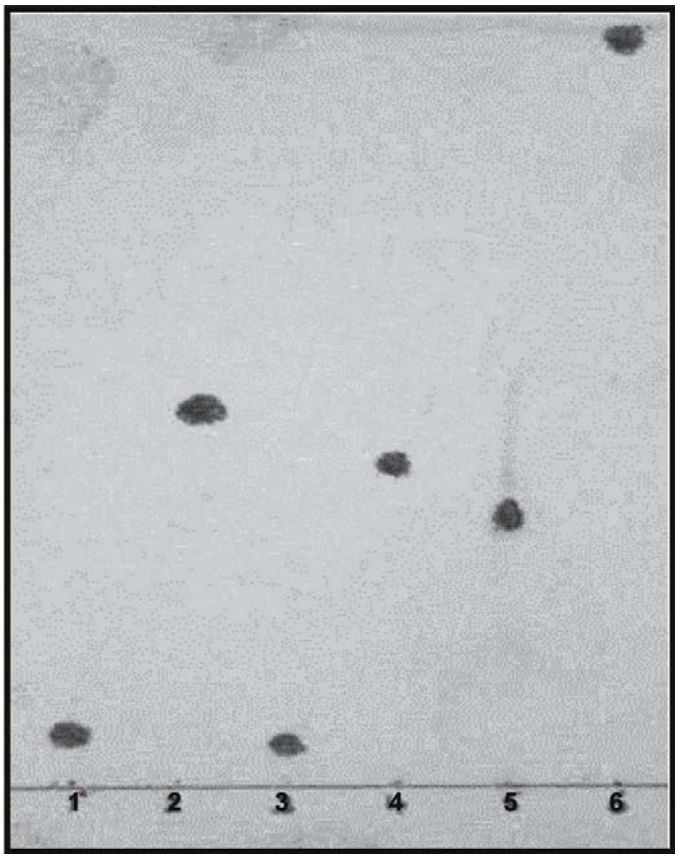

Fig 1: Thin layer chromatogram of P5C and (metabolically) related amino acids (1:Ornithine, Rf 0.07; 2:Glutamate, Rf 0.5; 3:Hydroxylysine, Rf 0.05; 4:Proline, Rf 0.42; 5:P5C, Rf 0.35). TLC was run, as described in text, along with the individual amino acids and the 'P5C-nihydrin derivative' (lane #6). Note the P5C synthesized appearing as single ninhydrin reacting spot on TLC. Also note the high Rf value of 'P5C-ninhydrin derivative' (# 6) moving almost with the solvent front as compared to amino acids and P5C per se.

spectral characteristics of the P5C -ninhydrin derivative (Fig 3A). As shown, in Fig. 2 the amu deduced for one of the major peaks correspond to the protonated species (amu: 114.1) of the parent compound (P5C: 113.1). Further, the mass spectra profile, as shown in Fig 2, also indicated the presence fragmented species of P5C, with molecular mass of 96 and 68.1 corroborating with mass fragmentation pathway depicted as 'inset'. The findings from these studies, apart from demonstrating the authenticity of P5C synthesized, also suggested the poor recovery (2.6%) of P5C (data not shown). The observed low recovery of the P5C could be due to the loss during the purification steps or due to uncontrolled oxidative reactions mediated by sodium metaperiodate which need to be addressed to enhance the recovery of P5C synthesis. Nevertheless, present study reaffirms the earlier method for the in house synthesis of P5C, which is not easily available from commercial sources, for any experimental

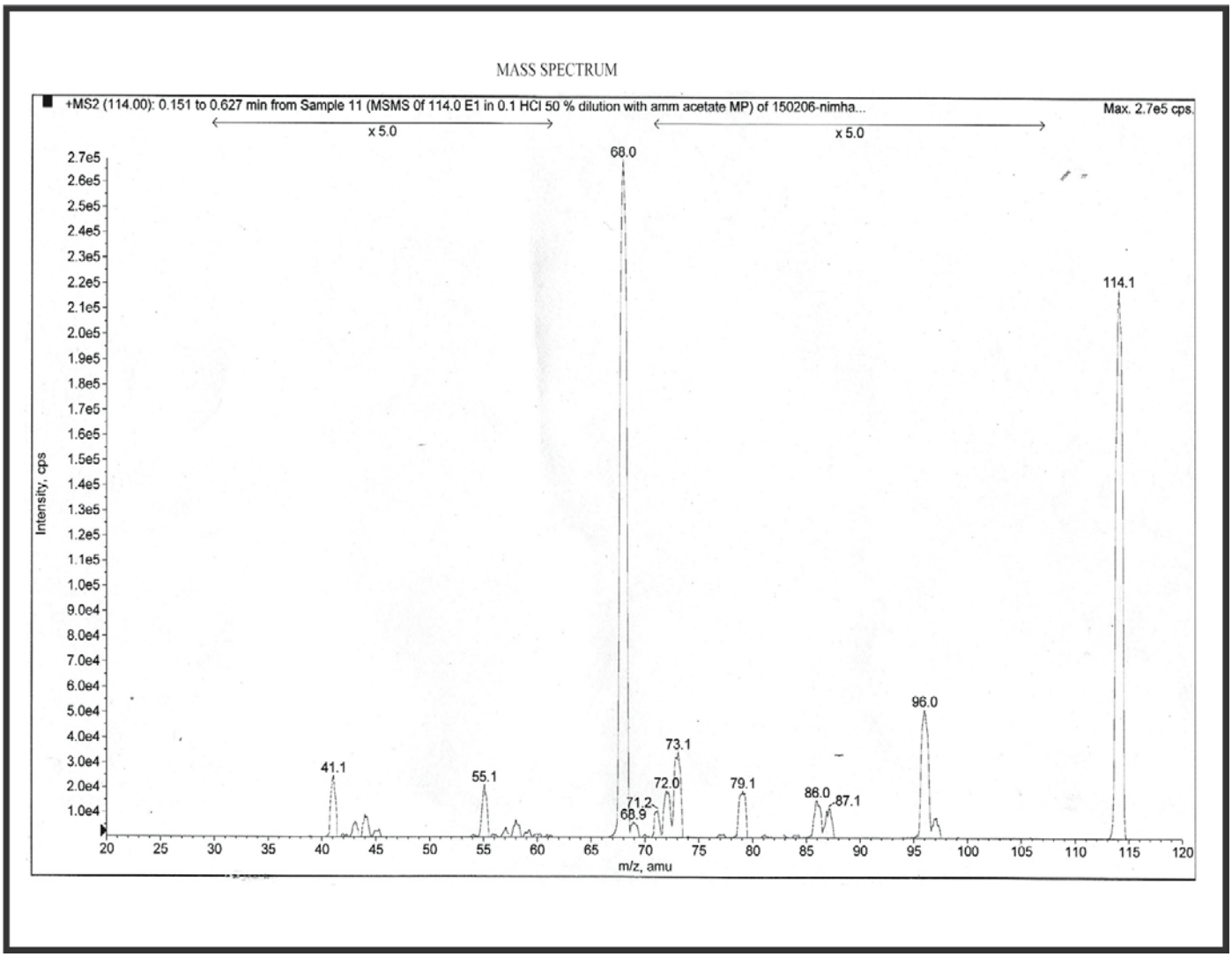

Fig. 2: Mass spectrometric profile of P5C synthesized and related molecular species. *Inset*: Proposed mass fragmentation pathway, with different mass fragments with respective masses. Note the peak corresponding to 114.1 amu, representing the protonated species of parent compound (P5C: with expected molecular mass of 113.1). Also seen are the two major peaks corresponding to fragmented species of C_5H_6NO and C_4H_6N corresponding to molecular mass of 96 and 68.1 respectively

studies. Further, we also noted that P5C is stable in acidic condition (1 N HCl) at 4-6 ºC for 5 months, which is contrary to the earlier report on its labile nature and the need to store at -80 ºC (11,12).

After having confirmed about the purity and authenticity of in-house synthesized P5C, upon scanning for UV absorption spectral characteristics, was found to have λ max at 210 nm (Fig 4), finding which is first to be reported on inherent absorption spectral characteristics of P5C. While the observation that P5C having λ max at 210 should enable to monitor the formation of this compound under defined conditions, such as the assay of OAT activity, the non specificity of absorption at this wavelength renders it unsuitable for the quantification of P5C in more complex biological materials. Hence, method for the assay of P5C based on specificity and sensitivity would augur the research on the role of P5C in health and diseases, including the assay of OAT activity in metabolic disorders. Under experimental conditions described for P5C-ninhydrin derivatisation, except for P5C, other amino acids failed to form insoluble chromogen confirming the specificity of insoluble ninhydrin derived chromogen to P5C. Thus, the present study, apart from confirming the insoluble nature of the P5C-ninhydrin derivative in aqueous medium and its solubility in ethanol as reported by the earlier investigators, also looked into the effect of pH on spectral characteristics of the chromogen, if any. Findings from the studies on the effect of pH on the spectral characteristics of

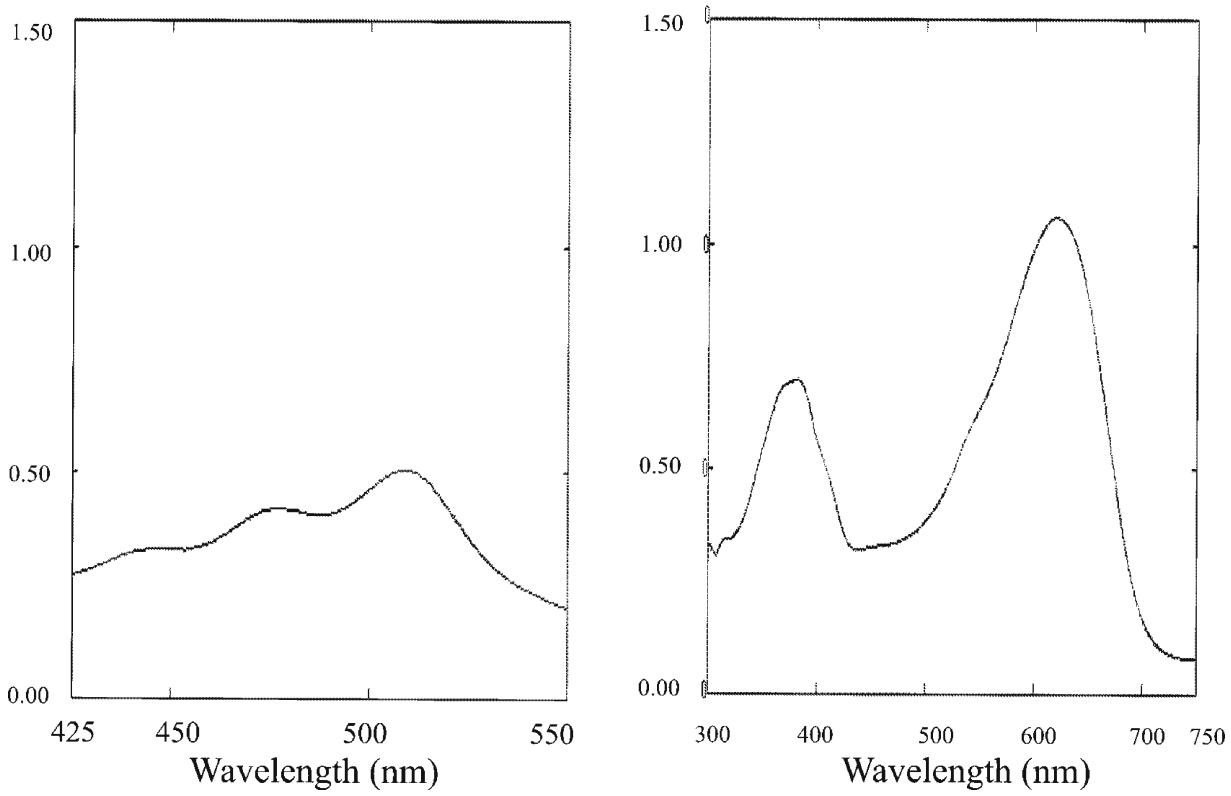

Fig. 3: Absorption spectral of characteristics of P5C-ninhydrin complex in ethanol (A) and in 50% (v/v) ethanolic buffer (50 mM bicarbonate buffer), pH:8.0 (B). Note the shift in λ max (620 nm) of P5C-ninhydrin complex at pH 8.0 as compared to λ max (510 nm) in ethanol only. Also note the increase in molar ε at pH 8.0 (1.96 x 10^5) as compared to1.65 x 10^4 in ethanol solution.

the P5C-Ninhydrin derivative demonstrated a significant shift λ max with an increase in Molar e at infrared region (Fig 4B). Thus, P5C-ninhydrin derived chromogen at pH ~ 7.5 - 8.0 had Molar ε of 1.96 x 10^5 at λ 620 nm, nearly twelve fold increase as compared to the earlier reported Molar e of 1.65 x 10^4 at λ 510 nm in ethanolic solution (11). The present finding

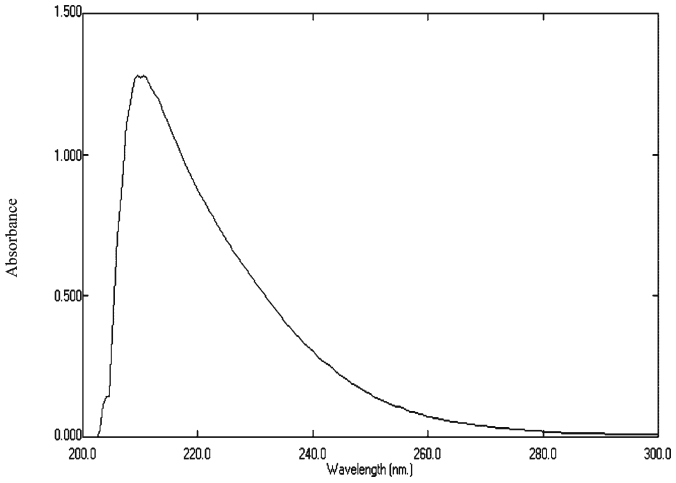

Fig.4: Absorption spectrum characteristics of P5C elute from Dowex 50W-8X resin. Note the λ max of P5C at 210 nm.

of enhanced Molar ε of P5C-ninhydrin chromogen in alkaline pH renders higher sensitivity to OAT assay method.

Using the newly established Molar ε of P5C-ninhydrin derivative in alkaline pH, OAT activity of WBC's and platelet lysate(s) were studied. The modified method of measuring the P5C-nihydrin derivative in alkaline pH renders the increased sensitivity for the assay the OAT activities from WBC's and platelet lysate(s) could be assayed. Of interest to note is the differential degree of enzyme activation from two sources., viz., WBC's (five fold increase) and platelets (nine fold increase) in presence of exogenous pyridoxal phosphate (PLP), though the basal activities (in the absence of PLP) of two tissue sources were found to be comparable (Table 1). The observed differential degrees of OAT activation by exogenous PLP from different tissue sources; viz., WBC's and platelets, appear to suggest their differential affinity to cofactor because of different isoenzymes or heteromers. These observations of differential stimulatory effect of PLP on OAT activity from different tissues opens new avenues to study the physiological relevance of these enzymes vis va vis interaction with PLP.

Table 1: Ornithine Amino Transferase activity in WBC's and Platelets lysate(s) of normal subjects in the absence or presence of pyridoxal phosphate.

Lysates (WBCs/ Platelets)	Specific Activity (nmol P5C formed /mg protein/minute)			
	WBC Lysates		Platelets Lysates	
	With PLP	With out PLP	With PLP	With out PLP
I	1.22	0.26	1.81	0.21
II	1.06	0.21	2.21	0.19
III	1.34	0.24	2.71	0.34
IV	1.95	0.31	2.94	0.29
V	1.10	0.18	1.71	0.16
Mean ± SD	1.33 ± 0.36	0.24 ± 0.08	2.28 ± 0.54	0.24 ± 0.08

Note: Considerable activation of OAT activity by exogenously added pyridoxal phosphate (PLP) in both WBCs and Platelet lysate(s). Also note the relatively higher specific activity of platelet lysate (s) in presence of (PLP) as compared to WBC's lysate, but comparable activity in both the lysates in the absence of PLP.

To summarize, the present study demonstrates the higher sensitivity for the assay of P5C, by establishing enhanced Molar e for P5C-ninhydrin derivative in alkaline condition.. The study, apart from demonstrating the application of modified method to monitor the OAT activity in tissue lysate(s) in clinical laboratories also reports for the first time the differential stimulatory effect of PLP on OAT activity from WBC's and Platelets. We propose, with appropriate pre-analytical considerations to prevent the degradation of P5C from biological resources, the method described to measure the P5C-ninhydrin derivative in alkaline pH may as well prove to be of use to establish reference interval of P5C in biological fluids and tissue sources to establish the relevance of P5C in health and disease conditions.

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