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IDENTIFICATION AND QUANTIFICATION OF AMINO ACIDS FROM PSORIATIC AND NORMAL EPIDERMIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

In this study, a modified fluorescence technique high performance liquid chromatography (HPLC) was adapted to separate the amino acids from the hydrolyzed keratin samples. These samples obtained from the epidermal layer of the normal and psoriatic human subjects. The keratin extracts were quantified in gram percentage of the dried skin and the amino acids concentrations were measured in μg/g, mean retention time (tR), slope value and the coefficient of determination (r2) of each eluted amino acid was calculated. The coefficients of variation for amino acid standards ranged from 0.12% to 0.28%, mean, standard deviation of peak area and coefficients of variation of peak area were calculated. From the normal hydrolyzed keratin protein fraction, 12 amino acids were determined and identified as aspartic acid, glutamic acid, asparagines, serine, glutamine, glycine, histidine, citrulline, arginine, β-alanine, tyrosine and valine. These amino acids were also determined in psoriatic samples while standard deviations (SD), standard error mean (SEM) and coefficient variation (CV%) of normal and psoriatic samples were also calculated. The higher concentration of amino acids in normal samples against psoriatic samples determined as glutamic acid 92.76±16.83/50.87±9.88, glutamine 198.05±18.74/19.74±3.74 while higher concentrations of amino acids was determined in psoriatic samples against normal samples such s asparagines 81.06±10.62/29.98±3.64/, arginine 164.42±35/46.14±8.46, tyrosine 214.38±29.61/59.64±8.82 and valine 169.7± 19.35/128.06±15.14. It is concluded that the absolute concentration of amino acids in psoriatic skin indicated a number of variations as compared to normal skin samples.

INTRODUCTION

Psoriasis is a chronic condition which results when skin cells over-produce and accumulate on the surface of the skin, producing red, scaly 'plaques' which may itch and bleed. It is thought to be genetic in origin and is a consequence of an abnormal inflamematory response in the skin. It has a significant negative impact on a patient's overall quality of life. Research is carried out on different options of severe psoriasis was linked with a number of serious medical conditions including cardiovascular disease, depression and cancer (Science Daily. 2008).

Psoriasis is described as inflamematory skin disease widely spreads all over the body mainly on scalp, knees,

axilla, abdomen, back and even on palms and soles along with the nails. The psoriasis arthritis is also a significant disease. It affects about 2 to 3% of the world population. The characteristic skin lesion is persistent, erythematous and scaly, reflecting infiltration and turnover of keratinocytes (Nikaein, et al., 2009). It has been reported that eruption of psoriatic lesions coincides with epidermal influx and activation of CD4+T cells (Baker et al., 1984). Cross reaction between proteins and human epidermal keratin is involved in the pathogenesis of psoriasis (Fadden et al., 1991) and (Valdimarrson et al., 1995). Keratin belongs to the family of coiled-coil protein structurally related to the M-proteins (Manjula et al., 1985). An excessive amino acid sequence homology with human epidermal keratin has been reported. Increased expression of this keratin has also been found in psoriatic patients (Leigh et al., 1995). In term of results, modern LC offers major advantages in convenience accuracy, speed and the ability to carry out difficult separation. The main advantage of bonded phase chromatography packing of material is that they are quite stable and can not be easily removed or lost during use **(**Peter, et al., 2007). Reversed phase bonded phase chromatography normally involves a relatively non-polar stationary phase (e.g. C-8 or C-18 hydrocarbon) used in conjunction with polar mobile phases to separate a wide variety of less polar solutes. High performance liquid chromatography for the separation of amino acids is widely used (Thomas and Michalke, 2003).

The aim of our study is to determine the fundamental factors which are responsible or play terrible role in increasing this disease. The polypeptide keratin possesses a chain of amino acids having the structural phenomena. The abundant diets of nucleoproteins also play a vital role in promoting psoriasis.

MATERIALS AND METHODS

Patients: One hundred fifty eight skin samples, 79 normal and 79 psoriatic, each group subdivided in 44 of males and 35 of females. The sufferers were mostly identified with psoriatic vulgaris type of the disease. Racial origins of the psoriatic patients and normal subjects were Pakistani. The patients were recruited from the out door patients (OPD) and the Skin wards of Liaquat University Hospital, Hyderabad and Jamshoro. The study was carried out under the supervision of the Department of Dermatology, Liaquat University Hospital. Clinical research ethics were properly followed. Stratum corneum and psoriasis scales obtained with six millimeter punch biopsies from the affected body sites of the patients while normal samples obtained through circumcision in males and wastage of skin pieces during minor operations of females. The skin biopsies preserved in 20% formaldehyde solution till further processed for the extraction of keratin and hydrolysis as reported by Martin and Syange (2007) and Bramhall, et al., (2008).

Sample preparation: The biopsies of the dried skin samples were suspended in to the mixture of Chloroform: Methanol (6:1 v/v) and kept for 60 minutes at room temperature. After 60 minutes the skin sample taken in chloroform and Methanol mixture was homogenized for 10 minutes with the three intervals. The contents were

filtered and samples were made free from fat and residue was saved for keratin extraction.

Sample pre treatment: Skin sample (0.76g) previously defatted, dried and cut in to slices was suspended in 200ml Tris - HCl buffer (pH 9) in 500ml volumetric flask and was kept on magnetic stirrer with magnetic bar. The sample suspended was continuously rotated for three hours then the contents were centrifuged for 20 minutes at 8000xg in refrigerated centrifuge. The supernatant was preserved as isolated keratin sample and preceded for further analysis. The amino acids separation was carried out by the modified procedure of Andrew, et al., (1964-2008).

Derivatizing protocol: A mixture of twenty one standard amino acids 1mg/ml of Aspartic acid, Glutamic acid, Asparagine, Serine, Glutamine, Glycine, Histidine,Threonine,Citrulline,Arginine, Alanine, Tyrosine, Methionine, Valine, Tryptophan, Phenylalanine, Iso-leucine, Leucine hydroxylysine, Lysine, Hydroxyproline were dissolved in aqueous solution. The mixture was added OPA reagent in equal volumes (25µl each). After 3 to 4 minutes, 25µl of the solution mixture was injected for the elution of amino acids.

Procedure: A mixture of twenty one standard amino acids (1mg/ml) comprising aspartic acid, glutamic acid, asparagines, serine, glutamine, glycine, histidine, threonine, citrulline, arginine, Alanine, tyrosine, methionine, valine, tryptophan, phenylalanine, isoleucine, leucine, hydroxylysine, lysine, hydroxyl -proline were dissolved in aqueous solution. 25µl OPA reagents in equal volumes were added for each reading. After holding 3 to 4 minutes 25µl of the solution mixture was injected for the elution of amino acids. The procedure adapted using high performance liquid chromatography (HLPC) with reversed phase column C18, guard ODS 3.9x150 mm obtained with serial no: 150531 p409, part no: 052885 from waters, Millipore, 3082f U.S.A. The amino acids were detected by using Hitachi F-1200 (Rheodyne Model 7125 injection valve) fluorescence spectrometer connected with Hitachi pump liquid chromatogram.

The excitation and emission wave lengths for OPA amino acids were adjusted at 340nm and 455nm respect-tively (Marshall, 2009). Laboratory data control (LDC) equipped with a manual inlet solvent switching four way valve, performed stepwise elution with two degassed solvent mixtures (solvent A, methanol: water 18:192 and solvent B, methanol: 0.05M potassium di-hydrogen phosphate, (pH 4.5) 3:2. Exactly 3.17ml (1ml/minute for 3 min and 10 sec) of solvent A allowed passing through the system, which has been equilibrated with solvent B thereafter. The sample 25 µl was injected afterwards to pass and elute through the system. Stepwise elution with flow rate 1ml / minute, solvent A: 10 min 50sec, solvent B: 26 min continued for high performance liquid chromatography analysis (Wayne et al., 1980; Qureshi, et al., 2009). The same conditions were applied for the detection of amino acids from hydrolyzed keratin samples of normal human epidermis and psoriatic patients (Neidl et al., 1989).

RESULTS

Three consequent readings of each concentration with their retention time were noted. Total retention time (tR)

(minutes) for all the eluted standard amino acids was within 32 minutes. The slope value and the value of coefficient of determination (r^2) of each eluted amino acid were determined. The standard amino acids separated from the mixture by HPLC were aspartic acid, glutamic acid, asparagines, serine, glutamine, histidine, glycine, threonine, citrulline, arganine, ß-alanine, tyrosine, methionine, valine, tryptophan, phenylalanine, isoleucine, leucine, hydroxylysine, lysine and hydroxyl-proline. The tR against each amino acid standard was within the range of 1:30 to 31:50. The r^2 of each amino acid standard was calculated within the range of 0.9987 to 1.000. The standard deviation for amino acid standards ranged from 0.03 to 0.15. The coefficient of variation (CV %) for amino acid standards ranged from 0.12% to 0.28%. Average peak area of amino acids ranged from 7.86 to 37.19 cm. The standard deviation (SD) of peak area of amino acid standards ranged from 0.07 to 0.64. While CV % of peak area of amino acid standards ranged from 0.36% to 2.78% (Figure 1). The concentrations of amino acids from normal and psoriatic human epidermal samples were calculated in (µg/g). Twelve amino acids determined from hydrolysated keratin protein fraction isolated from normal human epidermal extracts identified as aspartic acid, glutamic acid, asparagines, serine, glutamine, glycine, histidine, citrulline, arganine, ß-alanine, tyrosine, and valine. The higher concentrations were determined in glutamic acid, glutamine and valine as compared to the remaining amino acids (Figure 2). Twelve amino acids were determined from hydrolyzed keratin protein fraction isolated from psoriatic human epidermal extracts identified as aspartic acid, glutamic acid, asparagines, serine, glutamine, histidine, glycine, citrulline, arganine, β-alanine, tyrosine, and valine, respectively. The higher concentrations were determined in asparagines, arganine, tyrosine and valine as compared to the remaining amino acids (Figure 3). Average amino acid concentration (µg/g), Mean, SD, SEM and CV% of normal and psoriatic samples are mentioned in Tables 1 and 2. Significant variations and alterations were noted in HPLC of amino acids. Elevated concentrations of aspartic acid (55.17±8.93), glutamic acid (92.76± 16.83), glutamine (198.05±18.74), valine 128.06±15.14 and tyrosine 59.64 ±8.82 observed in normal samples while asparagines (81.06±10.62), arganine (164.42±35.11), tyrosine (214.38±29.61) and valine (169.7±19.35) showed maximum concentration in psoriatic samples. The calculations were followed by Descriptive Sampling Statistics. This site is a part of the JavaScript E-labs learning objects for decision making. Other JavaScript in this series are categorized under different areas of applications designed by Professor Hossein Arsham. http://home.ubalt.edu/ntsbarsh/Businessstat/otherapplets/Descriptive.htm. Our results indicate a clear manifestations and comparison between the diseased and non diseased subjects. Environmental, genetic inheritance and metabolic effects play an important role in promoting this disease.

DISCUSSION

The skin reflects its chemical constituents. The characteristics of toughness, elasticity and flexibility are due to the high concentration of keratin (a typical protein), which consists more

or less elongated peptide chain or a group of such chains. Keratin has a fibrous structure and present in the form of coiled (helical) peptide chains, with cross linkages between adjacent chains of the s-s formed from the amino acid cystine (Edward et al., 1970). The polypeptide composition of psoriatic epidermis is distinct but it is related to a regulatory function rather than to a structural gene defect. Keratohyalin granules are present in the granular, which give rise to the cornified envelop as a result of α-glutamyl-lysine cross links as reported by (Baden, et al., 2006). The biochemical approach was used to identify the amino acids in normal skin by HPLC (Henseler and Christophers, 1995). Protein is localized to the epidermis or stratum corneum (Ali et al., 2001). The keratinocytes are the source in psoriatic lesions (Harder et al., 2004). The amount isolated per gram of psoriatic scale material (10-25mg/g) was higher than that isolated from normal stratum corneum extracts (4-8 mg/g) (Harder et al., 2002). Peptides were found in skin samples from various body sites including keratinocytes, sweet ducts, and sebaceous glands of human skin (Fultons et al., 1997). Skin is free of signs of infections due to its contents such as lipids, acidic pH near 5, fatty acids, amino acids, salts and ambient temperature. Skin flora is that of an innate epithelial chemical defense shield in living organisms (Harder and Schorder, 2005).

CONCLUSION

It is concluded that amino acids identified and quantified in psoriatic skin by HPLC showed great difference in concentration as compared to normal skin. The keratin polypeptide and its amino acids alter in psoriasis whereas their adverse effects reflect on to the body in the sign of psoriasis. It is also observed that the proteins abundant in keratin (nucleoproteins) may be prohibited and their metabolic response may be watched during the treatment of psoriasis.

1. Aspartic acid
2. Glutamic acid
3. Asparagine
4. Serine
5. Glutamine
6. Glycine
7. Histidine
8. Threonine
9. Citrulline
10. Arganine
11.β-Alanine
12. Tyrosine
13. Methionine
14. Valine
15. Tryptophan
16. Phenylalanine
17. Isoleucine
18. Leucine
19. Hydroxylysine

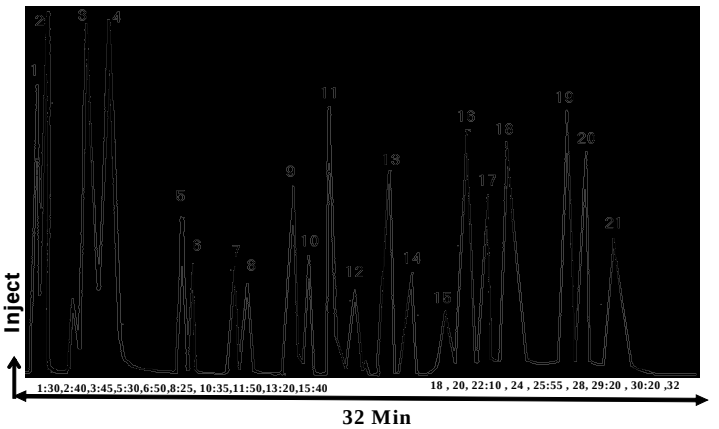


Figure-1: Chromatogram of o-phthalaldehyde derivative of 21 amino acids standard of physiological importance. Column 5 um C18 Waters Millipore U.S.A, reversed phase, Solvent A: Methanol + H_2O (18:192). Solvent B: KH_2PO_4 + CH_3OH (3:2). Fluorescent detector: Excitation 340: Emission 455, at flow rate of 1 ml/minute.

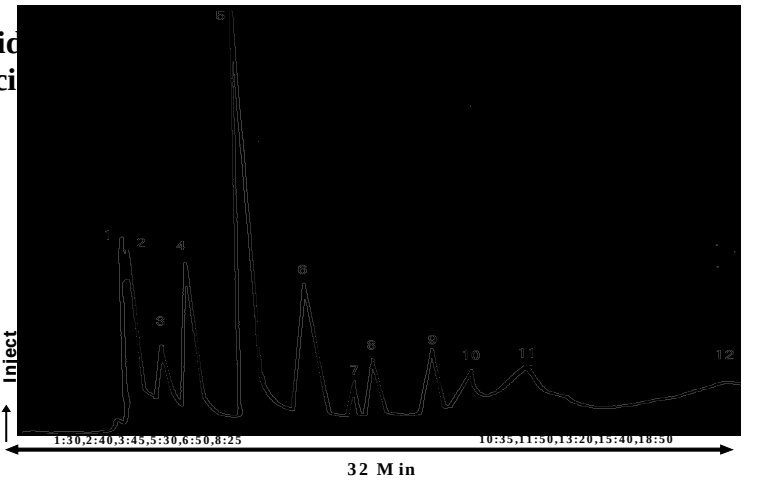
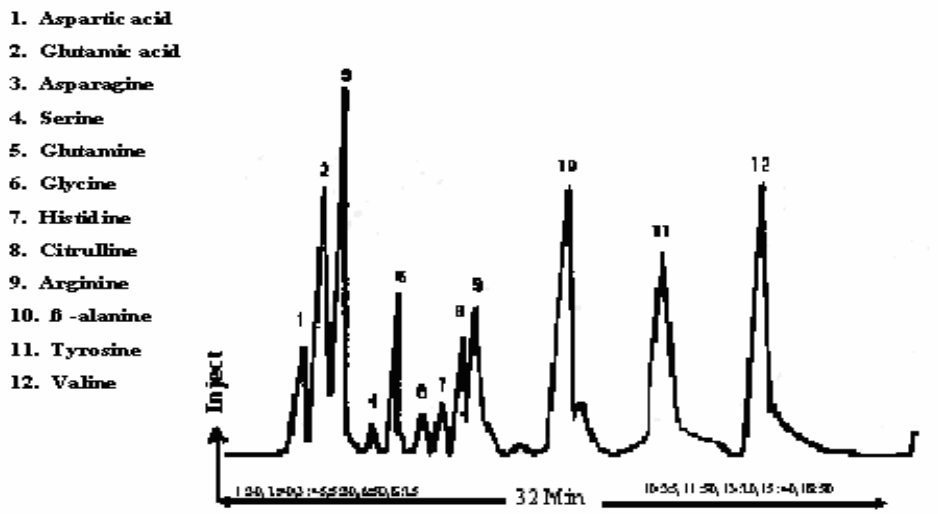


Figure-2: Chromatogram of Amino acids separated from Normal Skin Samples by High Performance Liquid Chromatography.



Chromatogram of Amino acids separated from Psoriatic Skin Samples by HPLC.

Figure-3: Chromatogram of Amino acids separated from Psoriatic Skin Samples by High Performance Liquid Chromatography

Table-1: Statistical data of Amino acids concentrations µg/g, calculated from hydrolyzed keratin samples isolated from normal human epidermis

No.	Amino acids	Mean± SEM	Standard Deviation	Coefficient of Variation
1	Aspartic acid	55.17±8.93	28.24	0.51
2	Glutamic acid	92.76±16.83	53.24	0.57
3	Asparagine	29.98±3.64	11.51	0.38
4	Serine	27.1±2.67	8.44	0.31
5	Glutamine	198.05±18.74	59.27	0.29
6	Glycine	49.2±9.83	31.10	0.63
7	Histidine	13.69±1.96	6.21	0.45
8	Citrulline	7.2±1.23	3.90	0.54
9	Arganine	46.14±8.46	26.75	0.57
10	β-Alanine	17.38±3.64	11.5	0.66
11	Tyrosine	59.64±8.82	27.91	0.46
12	Valine	128.06±15.14	47.89	0.37

Table-2: Statistical data of various amino acids concentrations µg/g, calculated from hydrolyzed keratin protein samples isolated from psoriatic human epidermis

No.	Amino-acids	Mean ± SEM	Standard Deviation	Coefficient of Variation
1	Aspartic acid	6.10±0.47	1.49	0.24
2	Glutamic acid	50.87±9.88	31.25	0.61
3	Asparagine	81.06±10.62	33.60	0.41
4	Serine	12.10±1.41	4.47	0.37

5	Glutamine	19.74±3.74	11.83	0.59
6	Glycine	17.98±7.34	23.22	1.29
7	Histidine	13.69±1.96	6.21	0.45
8	Citrulline	6.09±1.34	4.24	0.69
9	Arganine	164.42±35.11	111.03	0.67
10	β-Alanine	35.11±7.13	22.57	0.64
11	Tyrosine	214.38±29.61	93.65	0.43
12	Valine	169.7±19.35	61.19	0.36

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