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ANALYTICAL METHODOLOGY FOR ASSAYS OF SERUM TRYPTOPHAN METABOLITES IN CONTROL SUBJECTS AND NEWLY ABSTINENT ALCOHOLICS: PRELIMINARY INVESTIGATION BY LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

A rapid, isocratic assay for the determination of all major tryptophan metabolites in serum samples from control non-alcoholic subjects, recovered alcoholics and newly abstinent alcoholics is described. The sample preparation involves only precipitation of protein with sulfosalicylic acid. The complete liquid chromatographic analysis is short (25 min) and the sensitivity of amperometric detection permits the routine assessment of catabolites at the picogram level.

This preliminary longitudinal study of the basal and post-tryptophan load serum metabolites revealed a considerable scatter of experimental results for kynurenine and serotonin in all groups examined, probably owing to the clinical heterogeneity of the sample populations.

INTRODUCTION

The investigation of tryptophan (Trp) metabolism is a subject of considerable clinical and biochemical importance. The major quantitative route in the human body is the hepatic degradation of Trp to kynurenine (Ky) (Fig. 1, pathway A). The end-products of the second major pathway (Fig. 1, pathway B) are 5-hydroxyindoleacetic acid (5-HIAA) and 5-hydroxytryptophol (5-HTH). The two remaining, quantitatively minor, pathways leading to the formation of indoleacetic acid (IAA) (Fig. 1, pathway C) and indolepyruvic acid (Fig. 1, pathway D), have received relatively little attention.

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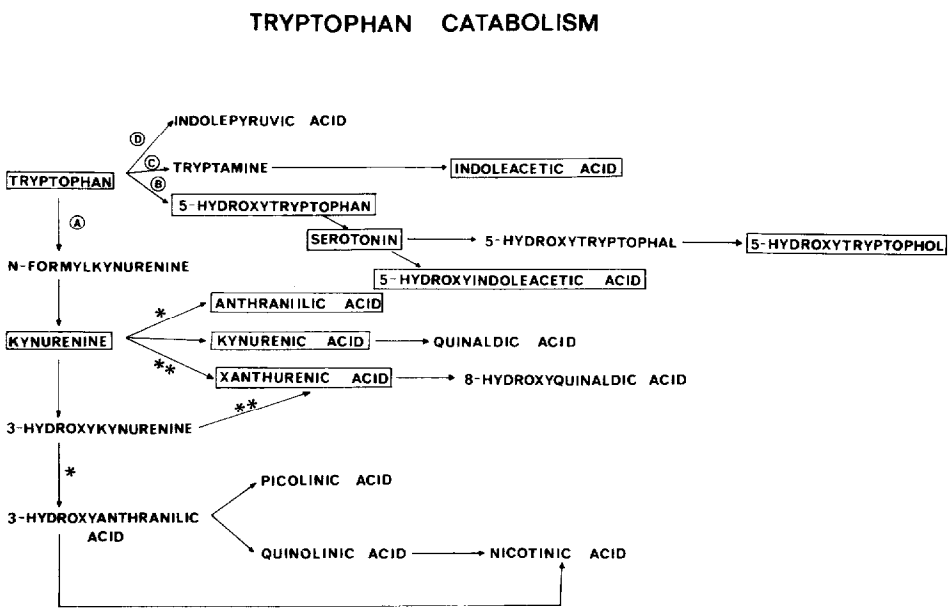

Fig. 1. Pathways of tryptophan catabolism (A, B, C and D). Metabolites in boxes are those to be measured in the serum of alcoholics and controls.* Reactions requiring pyridoxal phosphate;** reactions favored during pyridoxal phosphate deficiency.

Altered levels of Trp metabolites along the two major pathways have been observed in Hartnup disease[1], congenital tryptophanuria with dwarfism[2], depression[3], schizophrenia[4], Down's syndrome[5], carcinoid tumors[6], etc. In addition, there is clinical and biochemical evidence that Trp catabolism is affected in alcoholism, resulting in abnormal metabolite levels during chronic intoxication and immediately after withdrawal[7]. Reduced basal levels of urinary 5-HIAA among recently detoxified alcoholics[8], increased brain 5-HT synthesis[9] and enhanced platelet uptake of 5-HT[10] have been reported. Both the change in tryptophan pyrrolase activity and reduced protein synthesis have been invoked to rationalize these findings[11].

As Trp is an essential amino acid, the study of its biochemical degradation depends critically on the nutritional status, particularly in alcoholics. Hence it is very important to overcome the possible nutritional deficiency by administering a loading dose of Trp.

Different analytical methods have been employed for assays of Trp metabolites with varying degrees of success: thin-layer chromatography (TLC)[12], gas–liquid chromatography (GC) coupled with mass spectrometry[13], radioimmunoassay[14] and liquid chromatography (LC) with fluorescence[15] or electrochemical (EC) detection[16–18]. The last technique, particularly in its reversed-phase mode (RPLC), has proved to be the method of choice, as it affords rapid and efficient assays of these compounds at their endogenous levels with minimal sample pre-treatment.

As Trp metabolites include basic (serotonin, tryptamine, etc.), zwitterionic (Trp, 5-hydroxytryptophan, kynurenine, etc.) and acidic compounds (5-hydroxyindoleacetic acid, indoleacetic acid, indolepyruvic acid, etc.), most LC methods for

their concurrent determination employ gradient elution[16,18]. Although the re-equilibration of reversed phases during solvent change is rapid, the analysis time is nevertheless longer than in isocratic elution. Further, with highly sensitive detection devices, such as fluorimeters or EC detectors, baseline problems may arise.

To the best of our knowledge, none of the existing isocratic assays for Trp metabolites afford baseline resolution of all major Trp metabolites. Further, the widely differing chemical natures of these compounds have, in the past, precluded detection using a single detector. In a previous study we reported the concurrent use of EC detection and UV absorption[16]. We now report an improved isocratic HPLC analysis which permits highly sensitive determinations in less than 25 min. This method is ideally suited for longitudinal studies of Trp metabolites, such as those in which our laboratories have been involved over the past year.

EXPERIMENTAL

Apparatus

The HPLC apparatus consisted of a Model 112 Solvent Delivery Module (Beckman Instruments, Scientific Division, Berkeley, CA, U.S.A.), an Altex 210 injector with a 20-μl loop (Beckman Instruments), a Model 656 EC detector with an EA 1096 detector cell (Metrohm, Switzerland) and a Model 3390A electronic integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The EC detector cell, which operates on the wall-jet principle, consists of glassy carbon working and auxiliary electrodes and a silver–silver chloride reference electrode. Complementary characterization of some solutes was performed using a Jasco Uvidec-100-IV UV spectrophotometer (Varex, Rockville, MD, U.S.A.).

Chromatographic separations were performed using an Ultrasphere ODS (5 μm) column (150 × 4.6 mm I.D.).

Chromatographic conditions

The mobile phase for isocratic elution consisted of a 40 m*M* sodium acetate–citric acid buffer, containing 7% (v/v) acetonitrile. The pH of the eluent was adjusted to 4.5 with dilute potassium hydroxide solution. The mobile phases were always degassed by vacuum filtration prior to use. The solvent flow-rate was 0.7 ml/min and separations were performed at ambient temperature (21 ± 2°C). Solutes were detected amperometrically at +1.00 V *vs.* Ag–AgCl. This voltage afforded high sensitivity both for indolic compounds (for which this potential corresponded to the transport-limited portion of the potential–current plot) and for kynurenine.

Chemicals and reagents

All reference samples of Trp and related compounds were obtained from Sigma (St. Louis, MO, U.S.A.). Concentrated stock solutions (10 mg per 100 ml) of reference compounds were prepared in 40 m*M* acetate–citrate solution (pH 5.0) and were frozen and stored at −20°C, protected from light. Fresh calibration solutions were prepared each day by appropriate dilution of the stock solution.

All other chemicals and reagents were of the highest quality commercially available, and were used without further purification. Acetonitrile (RPE-ACS grade) was purchased from Carlo Erba (Milan, Italy).

Collection of blood samples

Alcoholics. Approximately 70 subjects, predominantly white, male and over 40 years old, admitted to the Alcohol Rehabilitation Program (ARP) at the Veterans Administration (VA) Hospital in White River Junction, VT, U.S.A., participated in the study. None of these patients exhibited serious medical complications, such as delirium tremens, hepatic failure or other alcohol-related problems. All patients were transferred to the ARP program only after stabilization. Patients in the ARP program routinely remained in the hospital for 3–4 weeks. Some subjects had liver damage, but most did not. The time since the last drink varied from 0 to 8 weeks, with an average of 1 week abstention prior to the first blood test. Subsequent analyses will determine whether hepatic function or whether time since the last drink significantly affected the results obtained with the present methodology.

Serum samples were collected ording to a protocol approved by the Institutional Review Board of the Darmouth Medical School and the Research and Development Committee at the VA hospital. All subjects investigated were given an oral dose of 2.0 g of L-Trp. This loading dose was chosen as it was reported not to induce Trp pyrrolase[19].

For baseline measurements, blood samples were drawn prior to the Trp loading. Freshly drawn blood was collected in tubes with no anticoagulant, and was allowed to clot spontaneously for 10–15 min at room temperature. Serum samples were separated from clotted blood elements and immediately frozen at −20°C. After the Trp loading, blood samples were taken from the same subjects at 2- and 4-h intervals following the administration of Trp. All subjects participated in a longitudinal study, in which they were followed for 18 months after the detoxification. Blood samples were collected successively from the same subjects at 1-, 3- and 6-month intervals after the beginning of the rehabilitation program. Twelve-month data had already been obtained on two subjects.

Control subjects. Control groups included non-alcoholic subjects (10) and recovered alcoholics with at least 3 years of sobriety (15). All control subjects received an oral dose of L-Trp and blood samples were collected under conditions identical with those for the alcoholics. Non-alcoholic controls had abstained from alcohol for 10 days prior to the challenge with oral Trp.

Preparation of serum samples prior to HPLC analyses

Initial experiments were conducted in order to find the best conditions for removal of serum proteins. Several methods described in the literature were tested: precipitation with 10% HCl, 3%, 6% and 12% $HClO_4$, 10% trichloroacetic acid, ultrafiltration through Amicon membrane cones (molecular weight cut-off 25,000), Sep-Pak filtration (C_{18} packing) and precipitation with 5% sulfosalicylic acid. All strongly acidic precipitants, which were mixed with serum in several volume proportions, afforded complete deproteinizations and similar solute recoveries (70–85%). However, subsequent freezing and thawing of extracts resulted in considerable sample decomposition. Further, even when these extracts were chromatographed immediately after acid precipitation, the chromatograms were more populated with compounds (other than those under investigation) than the sulfosalicylic acid extracts. This may indicate a certain degree of peptide and protein decomposition in highly acidic solutions, resulting in unnecessarily complex chromatograms. Ultra-

filtration with Amicon membrane cones was found to be an efficient method for protein removal[16]. However, in spite of efforts to pre-saturate the cones with the compounds of interest, some metabolites (particularly 5-HT) were not found in the extracts. This explains the absence of 5-HT in the ultrafiltrates analyzed using our previous method[16].

Next, an attempt was made to deproteinate and subsequently pre-concentrate the samples using the Sep-Pak C_{18} cartridges. The pre-concentration procedure, which was similar to that of Tonelli *et al.*[12], was found to be unsuitable as it resulted in incomplete retention of the metabolites (even at the pH of ionization suppression for acidic compounds). Further, subsequent elution from the cartridge required the use of approximately 50% organic modifier–buffer mixture. Injection of these eluates resulted in severe disturbances of the baseline (negative deflection) in EC detection, which interfered with the analysis of the early peaks.

The best recoveries and extract stability were obtained using a 5% solution of sulfosalicylic acid (serum–acid volume ratio 1:1). The procedure finally adopted was as follows. Freshly drawn blood samples (no anticoagulant) were allowed to clot for 15 min at room temperature. The serum was separated from clotted blood elements and 0.50 ml of serum + 0.50 ml of 5% sulfosalicylic acid were mixed in a Vortex mixer for 2 min. After centrifugation for 15 min at 1000 *g*, the supernatants were either analyzed immediately or stored at −20°C. Using this procedure, all compounds of interest were 90–98% recovered. The recovery studies were performed using a pooled serum sample as the matrix. The quantitative data are given in Table I. This method of deproteinization is sufficiently mild, and nevertheless highly efficient for protein removal (> 96%). The extracts, frozen at −20°C, were found to be stable for at least 1–2 weeks.

Attempts to protect the samples from eventual oxidation by addition of substances such as EDTA or ascorbic acid had an adverse effect on the chromatographic separation, as both compounds were detected, and thus interfered with the early peaks.

TABLE I

RECOVERY DATA FOR TRYPTOPHAN METABOLITES CARRIED THROUGH THE WHOLE SAMPLE PREPARATION PROCEDURE

All analyses were performed in triplicate using serum as the matrix, to which reference compounds were added at a concentration of 10 ng/l.

Compound	*Recovery (%)**
3-Hydroxykynurenine (3-HKy)	98 (1.9)
5-Hydroxytryptophan (5-HTP)	97 (1.6)
Kynurenine (Ky)	92 (1.7)
Serotonin (5-hydroxytryptamine, 5-HT)	96 (1.5)
3-Hydroxyanthranilic acid (3-HAA)	98 (1.6)
Tryptophan (Trp)	97.5 (1.7)
5-Hydroxyindoleacetic acid (5-HIAA)	94 (1.5)
5-Hydroxytryptophol (5-HTH)	90 (2.0)
Anthranilic acid (AA)	91 (1.9)

* The values in parentheses indicate the relative standard deviations (%).

Characterization of chromatographic solutes

Initial assignment of peak identities was made on the basis of retention times. However, the necessity to use an elevated oxidation potential in order to achieve a sufficient sensitivity for all Trp metabolites (particularly important for Ky) resulted in diminished detection selectivity. Thus additional characterization of peaks was necessary prior to the establishment of recognizable elution patterns. This was achieved by studying the elution characteristics of compounds in mobile phases of several pH values (4.0, 4.5, 5.0) and different organic modifier contents. In addition, hydrodynamic voltammograms were obtained for all compounds under study, and compared with those of the peaks in serum samples. Superimposability of the two voltammograms was taken as additional proof of peak identities.

Column stability

The chromatographic column used exhibited extremely high long-term stability under the analytical conditions described. Over 1200 assays were performed over 7 months without noticeable loss of column efficiency. In view of the high efficiency of the protein precipitation method, the use of guard columns was unnecessary. Only periodic cleaning of the entrance frit and purging of the column with 50 column volumes of pure acetonitrile after each 100 injections of serum extracts were practiced. Approximately 100 samples could be chromatographed consecutively without any carry-over of contaminants from previous injections.

RESULTS AND DISCUSSION

Prior to the analysis of serum samples, the chromatographic conditions were optimized for the isocratic separation of the major Trp metabolites (shown boxed in Fig. 1). A typical chromatogram of a synthetic mixture of reference compounds is shown in Figure 2. The pH of the mobile phase, and also the organic modifier content, were found to be particularly critical for 5-HIAA, 5-HTH and AA, as slight variations (0.1 pH unit or 1% difference in acetonitrile content) resulted in drastic changes in their retention times. All compounds except kynurenic acid could be detected amperometrically at +1.00 V *vs.* Ag–AgCl. The use of this potential was mandatory for sensitive detection of Ky. It is extremely important to maintain the ionic strength of the mobile phase sufficiently high, as even slight shifts in the working electrode potential may result in significant changes in sensitivity for Ky. Fig. 3 shows a potential–current curve for this compound, from which it is evident that the working potential of +1.00 V *vs.* Ag–AgCl did not correspond to the plateau region. This was not the case for the other compounds investigated. However, higher oxidation potentials were not used as they were found to decrease the electrode lifetime.

The detection of kynurenic acid, however, could not be achieved amperometrically and it was necessary to use a UV spectrophotometer (at 254 nm) connected in series with the EC detector.

The detection limits for all compounds under study are listed in Table II. The coefficient of variation determined in triplicate was 2.0% for the within-day determinations of peak areas and 3.3% for day-to-day measurements. The precision was poorer for a small number of samples obtained from sera with considerably lower protein content. This resulted in excess of sulfosalicylic acid, left unreacted after

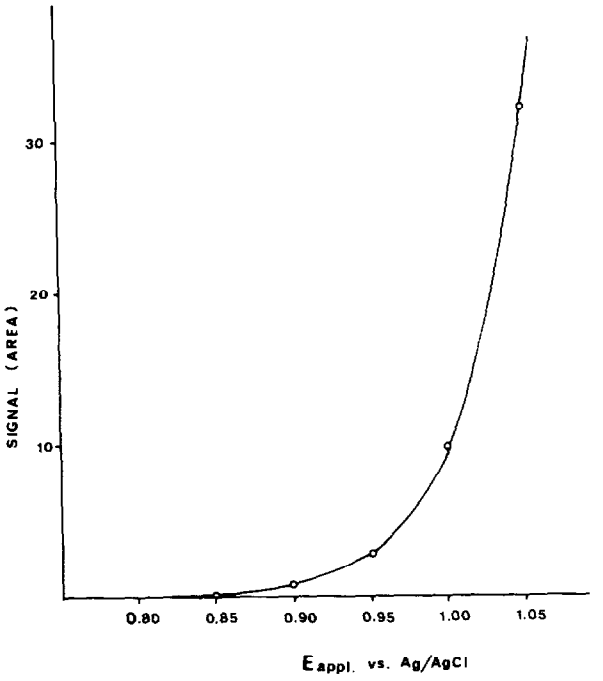

Fig. 2. Potential–current plot for kynurenine. It is evident that at the working potential of +1.00 V *vs.* Ag–AgCl the plateau is not attained. All other compounds under investigation were on the plateau region at this potential.

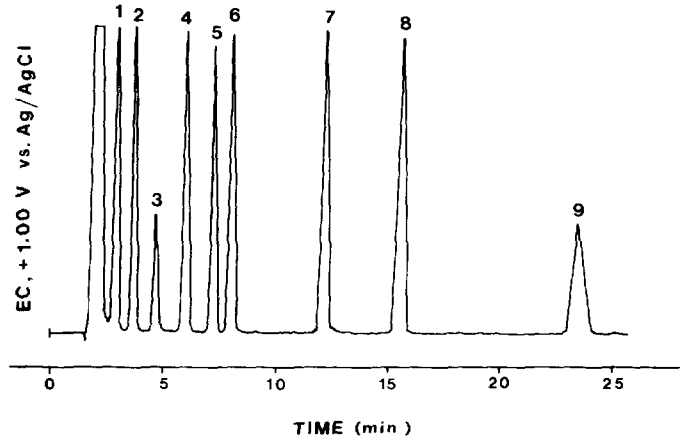

Fig. 3. Typical chromatogram of a synthetic mixture of reference compounds. Peak identities and amounts: 1, 3-HKy (10 ng); 2, 5-HTP (2.5 ng); 3, Ky (5 ng); 4, 5-HT (3.5 ng); 5, 3-HAA (5.8 ng); 6, Trp (2.25 ng); 7, 5-HIAA (2.5 ng); 8, 5-HTH (5.0 ng); 9, AA (5.0 ng). Chromatographic conditions: column, Ultrasphere ODS (5 μm) (150 × 4.6 mm I.D.); solvent, 40 m*M* sodium acetate–citric acid + 7% CH_3CN, pH 4.5; flow-rate, 0.70 ml/min; temperature, ambient; detection, amperometric at +1.00 V *vs.* Ag–AgCl; detection sensitivity, 1 μA full-scale; recorder sensitivity, 64 mV full-scale.

TABLE II

DETECTION LIMITS FOR TRYPTOPHAN METABOLITES UNDER INVESTIGATION

The signal-to-noise ratio was approximately 4:1.

Compound	*Detection limit (pg)*
3-Hydroxykynurenine	170
5-hydroxytryptophan	62
Kynurenine	120
5-Hydroxytryptamine (serotonin)	87
3-Hydroxyanthranilic acid	145
Tryptophan	56
5-Hydroxyindoleacetic acid	62
5-Hydroxytryptophol	125
Anthranilic acid	125

protein removal. In these instances it was necessary to decrease the concentration of sulfosalicylic acid to approximatcly 4% in order to safeguard the sample stability on storage. The detector responses were found to be linear for all compounds in the concentration range 50 pg to 100 ng.

Typical chromatograms of serum samples from a control, non-alcoholic subject taken prior to the oral loading with L-Trp (A), and at 2- and 4-h intervals following the Trp intake (B and C, respectively) are shown in Fig. 4. It should be noted that KA was detected spectrophotometrically at 254 nm. It is interesting that the increased Trp availability following the 2-g oral dose resulted primarly in preferential excretion of the metabolites along the Trp pyrrolase pathway (Ky and kynurenic acid). The preliminary results for the changes in metabolite levels along the serotonin and tryptamine pathways (5-HT, 5-HIAA, 5-HTP and KA) did not seem to be significant. It is evident, however, that the differences with respect to the basal metabolite levels were more pronounced 2 h after the Trp load. Analogous results were obtained for other non-alcoholic controls.

The analyses of a limited number of serum samples (15) from recovered alcoholics (at least 3 years of sobriety) showed chromatographic patterns similar to those of the control, non-alcoholic subjects, indicating that the possible abnormalities in Trp metabolism were reversible. However, more data are needed for a statistical evaluation.

The group of alcoholic subjects studied was composed of patients without serious liver damage who had abstained from drinking for 3–8 weeks. None of these patients received antabuse. Typical chromatograms illustrating the serum Trp levels at intake (A) and post-Trp load (B and C), and also after 1, 3 and 6 months, are shown in Fig. 5. The major changes observed among the different stages involved the two principal metabolites, Ky and 5-HT. The results for the metabolites in all populations exhibited considerable scatter, which can be explained in terms of the limited number of cases examined and their heterogeneous clinical histories.

The minor Trp metabolites, such as XA, AA and 5-HTH, were not consistently found in either the alcoholic or the control subjects. Thus the total number of cases

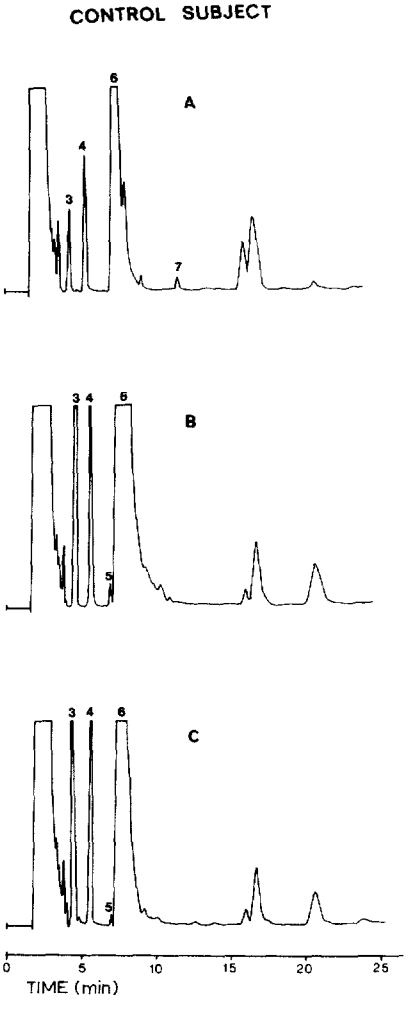

Fig. 4. Chromatograms of serum samples from a control, non-alcoholic subject. Volume of extract injected, 20 μl (corresponding to 10 μl of original serum). (A) Blood sample taken prior to the Trp loading. Solute identities and amounts: 2, 5-HTP (9.21 ng/l); 3, Ky (0.591 mg/l); 4, 5-HT (0.102 mg/l); 6, Trp; 7, 5-HIAA 0.029 mg/l); 8 5-HTH (9.1 ng/l). (B) Blood sample taken 2 h after Trp loading. Solute identities and amounts: 2, 5-HTP (8.4 ng/l); 3, Ky (2.46 mg/l); 4, 5-HT (0.158 mg/l); 5, 3-HAA (15.6 ng/l); 6, Trp; 8, 5-HTH (8.4 ng/l). (C) Blood sample taken 4 h after Trp loading. Solute identities and amounts: 3, Ky (1.59 mg/l); 4, 5-HT (0.180 mg/l); 5, 3-HAA (7.0 ng/l); 6, Trp; 7, 5-HIAA (0.044 mg/l). Chromatographic conditions as in Fig. 3.

was too small for statistically meaningful correlations, and additional data are being collected to obtain more information.

These preliminary results indicate that the Trp pyrrolase activity is diminished in alcoholics at intake and at 1 month, with the NAD pathway perhaps affected for as long as 3 months. A substantial number of the alcoholics were found to be pyridoxal phosphate-deficient, judging from the decreased production of 3-HAA.

The Trp hydroxylase pathway also appears to be altered in alcoholics, with

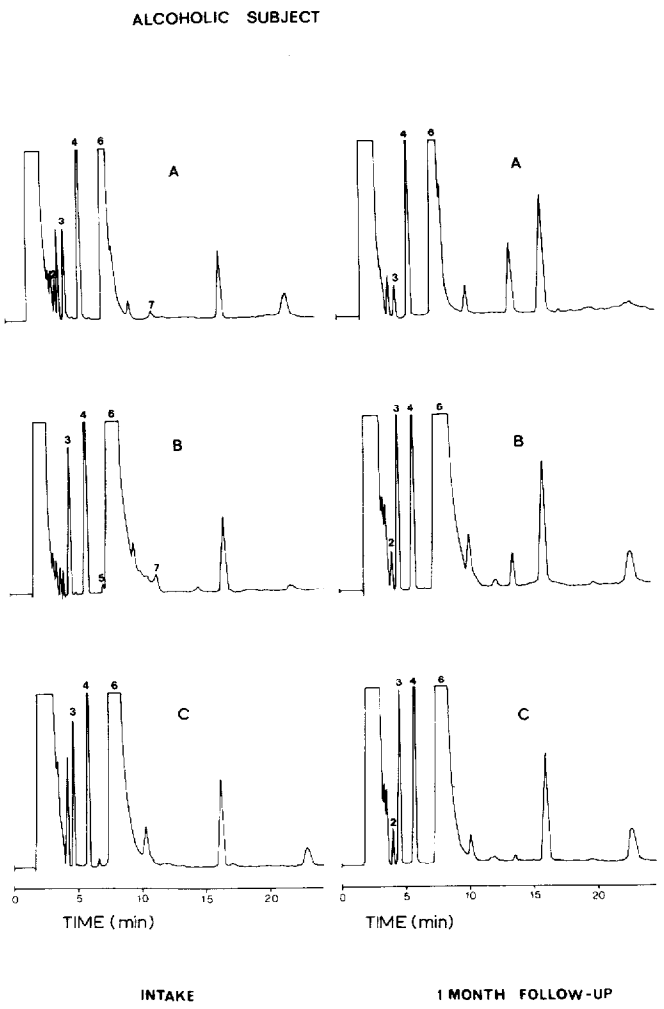

Fig. 5. Chromatograms of serum samples from an alcoholic subject. Volume of extract injected, 20 μl (corresponding to 10 μl of original serum). (A), (B) and (C), serum samples taken prior to, 2 h after and 4 h after Trp loading, respectively. The same procedure was repeated 1 month, 3 months and 6 months after the first assay. Solute identities and amounts were as follows. *Intake*: (A) 2, 5-HTP (14.5 ng/l); 3, Ky (0.740 mg/l); 4, 5-HT (0.301 mg/l); 6, Trp; 7, 5-HIAA (0.049 mg/l). (B) 3, Ky (1.22 mg/l); 4, 5-HT (0.301 mg/l); 6, Trp; 7, 5-IAA (0.035 mg/l); 8, 5-HTH (0.0228 mg/l). (C) 3, Ky (1.19 mg/l); 4, 5-HT (0.241 mg/l); 6, Trp. *1-month follow-up*: (A) 3, Ky (0.300 mg/l); 4, 5-HT (0.191 mg/l); 6, Trp. (B) 2, 5-HTP (0.026 mg/l); 3, Ky (1.49 mg/l); 4, 5-HT (0.220 mg/l); 6, Trp. (C) 2, 5-HTP (0.031 mg/l); 3, Ky (1.42 mg/l); 4, 5-HT (0.212 mg/l); 6, Trp. *3-month follow-up*: (A) 2, 5-HTP (0.0422 mg/l); 3, Ky (0.518 mg/l); 4, 5-HT (0.207 mg/l); 5, 3-HAA (3.06 ng/l); 6, Trp; 7, 5-MIAA (0.0192 mg/l). (B) 2, 5-HTP (0.0554 mg/l); 3, Ky (2.20 mg/l); 4, 5-HT (0.190 mg/l); 6, Trp. (C) 2, 5-HTP (0.0617 mg/l); 3, Ky (4.21 mg/l); 4, 5-HT (0.300 mg/l); 6, Trp; 7, 5-HIAA (0.0181 mg/l). *6-month follow-up*: (A) 2, 5-HTP (0.075 mg/l); 3, Ky (0.750 mg/l); 4, 5-HT (0.121 mg/l); 6, Trp; 7, 5-HIAA (0.023 mg/l). (B) 2, 5-HTP (0.098 mg/l); 3, Ky (2.10 mg/l); 4, 5-HT (0.153 mg/l); 6, Trp; 7, 5-HIAA (0.033 mg/l). (C) 3, Ky (1.72 mg/l); 4, 5-HT (0.075 mg/l); 6, Trp; 7, 5-HIAA (0.016 mg/l).

elevated baseline levels of serotonin at intake and at 1 month, but with below-normal baseline levels of 5-HIAA persisting even at 3 months. Consistently higher levels of 5-HTP were found among alcoholics at intake, at 1 and 3 months, and at the baseline and 2 and 4 h. This may indicate a possible shunting of 5-HT through this pathway.

It is also interesting that the KA levels in the alcoholics were elevated at the baseline at all occasions, and considerably decreased at 2 and 4 h at all time intervals.

In conclusion, owing to the complexity of the subject and the small sizes of the populations studied, the results of this preliminary study did not provide a direct unambiguous answer to all of the questions addressed. However, they demonstrate that differences exist between the two groups of subjects examined. We are hopeful that further quantitative work, including larger populations of normal non-alcoholic controls, and alcoholics during the first several weeks of detoxification, carefully classified with regard to liver damage, chronicity of alchoholism and family drinking history, will provide additional insight into this important topic. In addition, attempts are being made to study separately the peripheral and central Trp metabolism.

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