dria *(21)*. The deficit of these factors may cause excess hydroxyl-radical generation, leading to molecular and tissue damage. VE deficiency may affect nervous tissue in other ways, including overproduction of cytolytic phospholipids *(22)* and disturbance of brain monoamine metabolism *(23)*. Other roles of VE, such as modulation of necrosis κB and AP1 transcription factors and protein kinase C *(24)*, are probably essential for its neuroprotective effect.

Serum VE had rarely been investigated in unaffected family members of FA phenotype and was found to be within the reference interval in FA families *(4)* and decreased or within the reference interval in AVED families *(4, 5, 25, 26)*. In our study, absolute and lipid-adjusted VE was lower in the parents of AVED patients, who are obligatory heterozygous. These lower concentrations are probably attributable to a mild decrease of hepatic α-TTP activity in heterozygotes.

In conclusion, AVED is relatively frequent in Tunisia and probably in North African countries. Measurement of serum VE would permit recognition of AVED patients, in whom VE supplementation may be beneficial. This could be particularly helpful in developing countries where genetic testing is not available and can be expensive. However, genetic testing is still the gold standard because VE could also be low in FA patients with malabsorption or nutritional problems. Serum VE, and especially lipid-adjusted VE, may be useful for screening of heterozygotes in AVED families.

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More Rapid Method for Simultaneous Measurement of Tryptophan and Kynurenine by HPLC, *Andreas Laich,[1] Gabriele Neurauter,[1,2] Bernhard Widner,[1] and Dietmar Fuchs[1,2*]* ([1] Institute for Medical Chemistry and Biochemistry, Leopold Franzens University, A-6020 Innsbruck, Austria; [2] Ludwig Boltzmann Institute for AIDS-Research, A-6020 Innsbruck, Austria; * address correspondence to this author at: Institute for Medical Chemistry and Biochemistry, University of Innsbruck, Fritz Pregl Strasse 3, A-6020 Innsbruck, Austria; fax 43-512-507-2865, e-mail dietmar.fuchs@uibk.ac.at)

The essential amino acid L-tryptophan is important in nitrogen balance and the maintenance of muscle mass and body weight in humans *(1)*. Moreover, tryptophan is the precursor for the biosynthesis of the neurotransmitter serotonin (5-hydroxytryptamine). Insufficient availability of tryptophan may increase susceptibility for mental depression *(2)*. On activation of cellular immunity, the T-cell-derived cytokine interferon-γ stimulates the enzyme indoleamine-(2,3)-dioxygenase (IDO) in various cells *(3, 4)*. IDO catalyzes the initial step of tryptophan catabolism within the biosynthetic pathway of nicotin-

amide dinucleotides, and *N*-formyl-kynurenine is formed as a first intermediate.

The kynurenine-to-tryptophan ratio has been a sensitive estimate to monitor the activation status of IDO and of cellular immunity both in vivo and in vitro *(3, 5)*. In patients, a decrease in serum tryptophan and a parallel increase of kynurenine attributable to IDO activation is found in various diseases associated with T-cell activation, such as viral infections, autoimmune disorders, and malignant diseases *(3, 5–8)*. More recently, activation of IDO in monocytes/macrophages was found to interfere with the proliferative capacity of T cells in response to antigenic stimulation by the withdrawal of tryptophan *(9)*. This finding has attracted immunologic researchers especially to explore the possible involvement of IDO in tolerance induction and in diseases that are associated with acquired immunodeficiency.

We have described a reverse-phase HPLC method to quantify serum concentrations of kynurenine and tryptophan in parallel with use of 3-nitro-L-tyrosine as an internal standard *(10)*. Here we report an optimized protocol that uses a shorter HPLC column and a different elution buffer, allowing faster throughput of samples without loss of specificity and sensitivity.

The chromatographic system applied is identical with the one described previously *(10)*. In brief, a Varian 9010 solvent delivery system was used. Sample injection (25 μL) was controlled by a Jasco AS-950 autosampler with a cooling unit (4 °C), and a 10-μL loading loop was used. Kynurenine and 3-nitro-L-tyrosine were measured by a Jasco UV 975 detector at 360 nm; tryptophan was detected by a fluorescence detector (Model ProStar 360; Varian) at an excitation wavelength of 286 nm and an emission wavelength of 366 nm. Both detectors were connected in series to allow simultaneous measurements. To control the set-up and for peak quantification, Borwin 1.5 and MS Excel software was used.

Samples were prepared as described previously *(10)*. Briefly, before each HPLC analysis, 1000 μL of albumin-based calibration mixture was prepared containing 50 μmol/L tryptophan (Serva) and 10 μmol/L kynurenine (Sigma). A frozen serum pool was used as external standard. For sample preparation, the calibration mixture, the pool serum, or samples were equally treated as follows: 100 μL of the appropriate specimen was diluted with 100 μL of 50 μmol/L 3-nitro-L-tyrosine (Sigma), and after the addition of 25 μL of 2 mol/L trichloroacetic acid (Merck), the reaction vials were immediately vortex-mixed. Samples were centrifuged at 12 000 *g* for 6 min at room temperature to precipitate and separate the protein. Supernatants (180 μL) were transferred into microsampling vials placed in screw-cap vials (both from Chromacol) suitable for the autosampler used. The concentrations of components were calculated according to peak heights and were compared with 3-nitro-L-tyrosine as the internal standard.

For separation, we used a reverse-phase LiChroCART 55-4 cartridge (Merck), 55 mm in length, filled with Purosphere STAR RP_{18} (3-μm grain size; Merck) together with a reverse-phase C_{18} precolumn (Merck). The flow rate was 0.9 mL/min at room temperature. After a chromatographic session was completed, once per day the system was rinsed with a gradient to/from pure methanol and the precolumn was replaced.

Because the column used was shorter than the column used in our previous study (244 mm) *(10)* and to achieve resolution of peaks, it was necessary to change the elution buffer to 15 mmol/L acetic acid–sodium acetate (pH 4.0; Merck), containing 27 mL/L acetonitrile (Merck). Retention times were 2.3 min for kynurenine and 3.3 min for 3-nitro-L-tyrosine (Fig. 1 top chromatogram); tryptophan eluted after 4.3 min (Fig. 1, bottom chromatogram). One single chromatographic run was completed within 7 min.

To test the reproducibility of the method, we aliquoted one serum pool into 20 samples and stored them at −20 °C. Between-day variation was assessed by measuring these aliquots on 20 consecutive days, using one freshly thawed sample every day. Tryptophan and kynurenine concentrations were 72.8 ± 1.2 μmol/L (mean ± SD; CV = 1.7%) and 2.4 ± 0.1 μmol/L (CV = 4.2%), respectively. Another serum pool was aliquoted into 10 samples, all of which were measured on 3 consecutive days. Between each of the series, samples were stored at 4 °C in the dark overnight. The within-day variation of tryptophan concentration was 65.8 ± 0.7 μmol/L (CV = 1.0%) and 65.6 ± 1.0 μmol/L (CV = 1.4%), respectively. The variability of kynurenine concentrations was 2.7 ± 0.04 μmol/L (CV = 1.5%) and 2.8 ± 0.04 μmol/L (CV = 1.4%).

To test the linearity of the system, we diluted a third serum pool, containing 91.1 ± 3.6 μmol/L tryptophan (mean ± SD; CV = 1.0%) and 6.0 ± 0.3 μmol/L kynurenine (CV = 4.9%), with H_2O to 1:2.5, 1:5, 1:10, 1:50, and 1:100. Each dilution was measured in quadruplicate; the mean tryptophan and kynurenine concentrations and standard deviations were determined for all dilutions and compared with the concentrations calculated from the undiluted samples. Linear regressions (calculated vs measured) were determined for both analytes, revealing a slope of 0.996 for tryptophan and 0.999 for kynurenine. The linear regression coefficients (r^2) were >0.998.

Recovery was examined using serum specimens (50 μL each) from a reference pool containing 62.2 μmol/L tryptophan and 2.3 μmol/L kynurenine. Specimens were supplemented with 50 μL of 125 or 100 μmol/L tryptophan (final concentrations, 93.6 and 81.1 μmol/L tryptophan) and 50 μL of 12.5 or 10 μmol/L kynurenine (final concentrations, 7.4 and 6.2 μmol/L kynurenine), respectively. Each supplementation was performed in triplicate and yielded recoveries of 99.3% ± 0.4% (mean ± SD) for tryptophan and 102.3% ± 1.0% for kynurenine.

In sera obtained from 40 healthy blood donors (20 males and 20 females; age, 41 ± 11 years), tryptophan and kynurenine concentrations were 67 ± 15 μmol/L and 1.9 ± 0.5 μmol/L, respectively. These mean concentrations compared well with those described previously *(10)*. The same specimens were also analyzed by the previously described HPLC method, using a longer column and

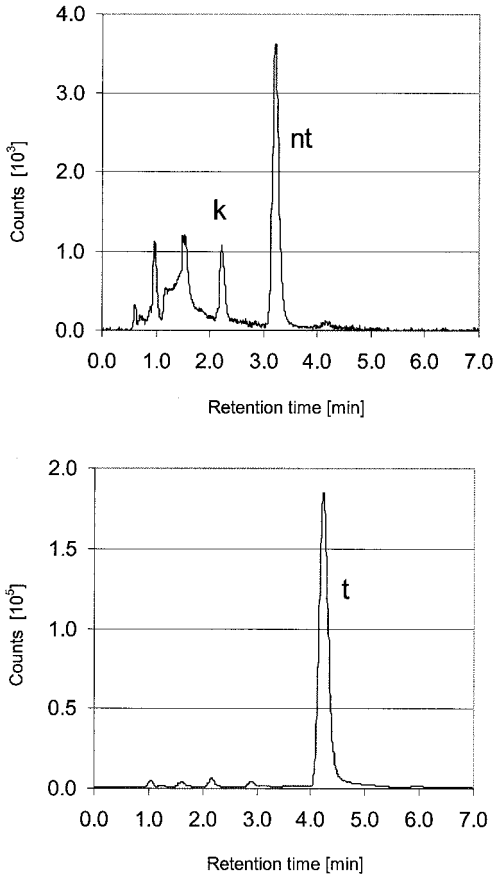

Fig. 1. Typical chromatograms showing simultaneous measurement of tryptophan (*t*) and kynurenine (*k*) with 3-nitro-L-tyrosine (*nt*) as internal standard.

Retention times for kynurenine and nitrotyrosine were 2.3 and 3.3 min, respectively; both substances were detected by their ultraviolet absorbance at 360 nm. Tryptophan eluted at 4.3 min (fluorescence detection was as follows: excitation wavelength, 266 nm; emission wavelength, 366 nm). In this specimen, tryptophan and kynurenine concentrations were 71.5 and 2.17 μmol/L, respectively.

phosphate buffer (15 mmol/L, pH 6.4) for elution. The linear regression coefficients (r^2) were 0.981 for tryptophan (slope = 1.004) and 0.905 for kynurenine (slope = 1.21).

The presented method is well suited for high throughput of samples. Approximately 150 specimens can be measured with one chromatographic system per day. On the one hand, it allows measurement of tryptophan and kynurenine concentrations with high sensitivity. Determination of tryptophan may be especially important for psychiatric clinics when the background of increased susceptibility for mental depression needs to be characterized more precisely. On the other hand, the parallel measurement of kynurenine concentrations will allow ruling out whether low tryptophan is attributable to insufficient dietary intake of the essential amino acid tryptophan and allows an estimate of IDO activity in vitro and in vivo when the kynurenine-to-tryptophan ratio is calculated. The sensitivity of the method can be easily enhanced by increasing the injection volume to 100 μL, which is necessary when analyzing cerebrospinal fluid or cell culture supernatants in which kynurenine concentrations are one order of magnitude lower than in serum or plasma.

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Methemoglobin Interferes with the HemoCue B-Glucose Analyzer, *Patrick L. M. Lynch* and Maurice J. O'Kane* (Department of Clinical Chemistry, Altnagelvin Area Hospital, Glenshane Rd., Londonderry BT47 6SB, United Kingdom; *author for correspondence: fax 44-2871-313036, e-mail mlynch@alt.n-i.nhs.uk)

During the investigation of a neonate with NADH-methemoglobin (NADH-MetHb) reductase deficiency (baseline MetHb, 28%), we noticed disparate readings between the ward-based, whole-blood HemoCue B-Glucose Analyzer (HemoCue AB) and the main laboratory plasma glucose method (glucose oxidase) performed on a Synchron CX7 Delta analyzer (Beckman Coulter). The HemoCue results suggested profound hypoglycemia, which was out of keeping with the clinical status of the patient, whereas the Synchron CX7 Delta results were consistently within the pediatric reference interval. These findings were contrary to the previously documented overestimation of the HemoCue analyzer at low glucose concentrations *(1–3)*. Because the HemoCue analyzer *(1–11)* uses a mutarose–glucose dehydrogenase enzyme system to produce a colored formazan, which is then quantified photometrically, the possibility of spectral