Simultaneous Measurement of Serum Tryptophan and Kynurenine by HPLC, *Bernhard Widner, Ernst R. Werner, Harald Schennach,[1] Helmut Wachter, and Dietmar Fuchs** (Inst. of Med. Chem. and Biochem., Univ. of Innsbruck, Fritz Pregl Str. 3; [1]Central Inst. of Blood Transfusion and Immunol., A-6020 Innsbruck, Austria; *author for correspondence: fax ++43-512-507-2865, e-mail Dietmar.Fuchs@uibk.ac.at)

L-Tryptophan is an essential amino acid that is required for the biosynthesis of proteins and is the precursor for several important biological compounds, e.g., 5-hydroxytryptamine (serotonin). In an alternative reaction series, the so-called kynurenine pathway, tryptophan is catabolized to form nicotine amides and the vitamin niacin *[1]* as end products. A cytokine-inducible indoleamine-(2,3)-dioxygenase (IDO) catalyzes the first step of tryptophan degradation *[2–5]*, forming the intermediate kynurenine. Interferon-γ has been shown to be a potent stimulating cytokine for IDO in vitro *[6, 7]*. Similarly in vivo, a decrease of serum tryptophan and an increase of kynurenine in parallel was described *[8–12]*, indicating an enhanced cytokine-induced degradation of tryptophan, when the cellular immune system is activated. In particular, the ratio of kynurenine to tryptophan seems to be a sensitive indicator for interferon-γ-induced tryptophan degradation and therefore for an activated immune system *[10, 13]*. Thus, simultaneous measurements of kynurenine and tryptophan, allowing calculation of the ratio, enables indirect examination of endogenous interferon-γ formation.

We describe a new HPLC method to determine the amounts of kynurenine and tryptophan in serum simultaneously with use of an external albumin-based calibrator and an internal calibrator. The human subjects involved within the scope of this method development correspond to the ethical standards of our institutions' responsible committee.

L-Tryptophan was obtained from Serva, L-kynurenine and 3-nitro-L-tyrosine were from Sigma, and potassium phosphates and acetonitrile for the HPLC elution buffer were from Merck. All chemicals used were of analytical grade. The HPLC pump was a Model 9010 (Varian) controlled by a DS 654 data system. Sample collection was performed by an autosampler (AS-950, Jasco) with a 100-μL sample loop and a cooling unit (4 °C). For separation, reversed-phase cartridges LiChroCART RP_{18} columns (244 mm length, 5 μm grain size) from Merck were used. Tryptophan was detected by a fluorescence detector (Hewlett Packard, Model 1046A) at an excitation wavelength of 285 nm and an emission wavelength of 365 nm. A Shimadzu SPD-6A UV-detector (Shimadzu) in flow stream series connection was used for detection of both kynurenine and nitrotyrosine at a wavelength of 360 nm.

The elution buffer was a degassed potassium phosphate solution (0.015 mol/L, pH 6.4) containing 27 mL/L acetonitrile. RP_{18} precolumns (Merck) were used to protect the column from apolar ingredients of biologic material. They were replaced daily after measurements of approximately 30–40 serum specimens. The HPLC cartridge was rinsed with a gradient from water to methanol and back within 40 min each day. Analyses were carried out at a flow rate of 0.8 mL/min and a temperature of 25 °C.

Frozen serum specimens were thawed at room temperature. Two-hundred microliters of serum were diluted with 200 μL of potassium phosphate buffer (0.05 mol/L, pH 6.0) containing the internal calibrator 3-nitro-L-tyrosine (100 μmol/L). Protein was precipitated with 50 μL of trichloroacetic acid (2 mol/L). The capped tubes with the precipitate were immediately vortex-mixed and centrifuged for 10 min at 13 000*g*. One-hundred-fifty microliters of the supernatants were transferred into microvials (Chromacol) and placed into the autosampling device.

The external calibrator was prepared from freshly thawed stock solutions of tryptophan and kynurenine (1 mmol/L in bidistilled water, stored at −20 °C) and albumin (70 g/L, which corresponds to the average physiological protein content in human serum). Fifty microliters of tryptophan, 10 μL of kynurenine, and 940 μL of albumin stock solution were mixed together. Aliquots of 200 μL of calibrator preparation were then treated in the same way as the serum specimens.

Before measurements were started, two serum samples were preinjected, preconditioning the rinsed column, and two external calibrators were injected as preruns followed by the analytical runs. After each block of six sample specimens, one external calibrator was measured.

Peak area counts were used to calculate the concentrations. Tryptophan and kynurenine were referred to nitrotyrosine. Thus, the ratios of tryptophan per nitrotyrosine and kynurenine per nitrotyrosine for the external calibrator were calculated as well as for the sample, leading to the final results. The reproducibility of the system was controlled by nitrotyrosine counts. Considering the total standard deviation of the method, variation of nitrotyrosine <5% was tolerated.

Linearity was tested by diluting serum with bidistilled water down to a concentration of 0.09 μmol/L for kynurenine and 0.06 μmol/L for tryptophan and by supplementing with either tryptophan or kynurenine in the following way: A 200-μL specimen with 57.1 μmol/L tryptophan and 2.87 μmol/L kynurenine was supplemented with volumes of 200 μL of phosphate buffer solution (pH 6) containing 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 nmol of kynurenine and, in another series, 5, 10, 15, 20, 25, and 30 nmol of tryptophan. The corresponding recoveries were 98.9% (tryptophan) and 103.3% (kynurenine).

Superposition of the dilution and the supplementing series showed linearity throughout the measured concentration ranges (not shown) of 0.06–222 μmol/L for tryptophan and 0.09–9.84 μmol/L for kynurenine. The detection limits were measured at signal-to-noise ratios of 3, referred to the mean signal of a blank baseline.

Figure 1 shows representative chromatograms of serum and albumin-based calibrator. The retention time was 12.2 min for kynurenine, 16.8 min for nitrotyrosine (both by UV detection), and 27.4 min for tryptophan (fluorescence

detection). Flow rates >0.8 mL/min or temperatures >25 °C turned out to be less useful, as other compounds tended to coelute with kynurenine.

Within-day and between-day imprecisions (CVs) were evaluated from three different albumin-based preparations containing 20.7, 59.4, and 104 μmol/L tryptophan and 2.11, 3.30, and 7.6 μmol/L kynurenine (Table 1). The specimens were prepared on 1 day and were frozen in aliquots of 200 μL at −20 °C.

In the sera of 49 healthy blood donors (28 men, 21 women) the serum metabolites were 73.0 μmol/L ± 14.9 (tryptophan), 1.92 μmol/L ± 0.58 (kynurenine), and 26.9 ± 8.10 (ratio). Tryptophan and kynurenine concentrations were 15% higher in men than in women ($P < 0.05$). The range of the data was in accordance with concentrations measured earlier in a small number of healthy controls *[13]*, applying HPLC chromatography with on-line deproteinization *[14]*. There was no evidence of age dependency in the examined sera.

Table 1. Between-day (n = 20) and within-day (n = 7) imprecisions of a high-, medium-, and low-range sample.

Imprecision	Tryptophan	Kynurenine
Between-day		
High	104 ± 3.24 (3.10)	7.6 ± 0.32 (4.21)
Medium	59.4 ± 2.78 (4.68)	3.30 ± 0.19 (5.80)
Low	20.7 ± 1.2 (5.78)	2.11 ± 0.27 (12.81)
Within-day		
High	103 ± 0.89 (0.86)	7.43 ± 0.14 (1.88)
Medium	62.5 ± 0.87 (1.39)	3.22 ± 0.18 (5.57)
Low	21.1 ± 0.20 (0.96)	2.28 ± 0.13 (5.71)

Measurements are mean (μmol/L) ± SD (percent CV).

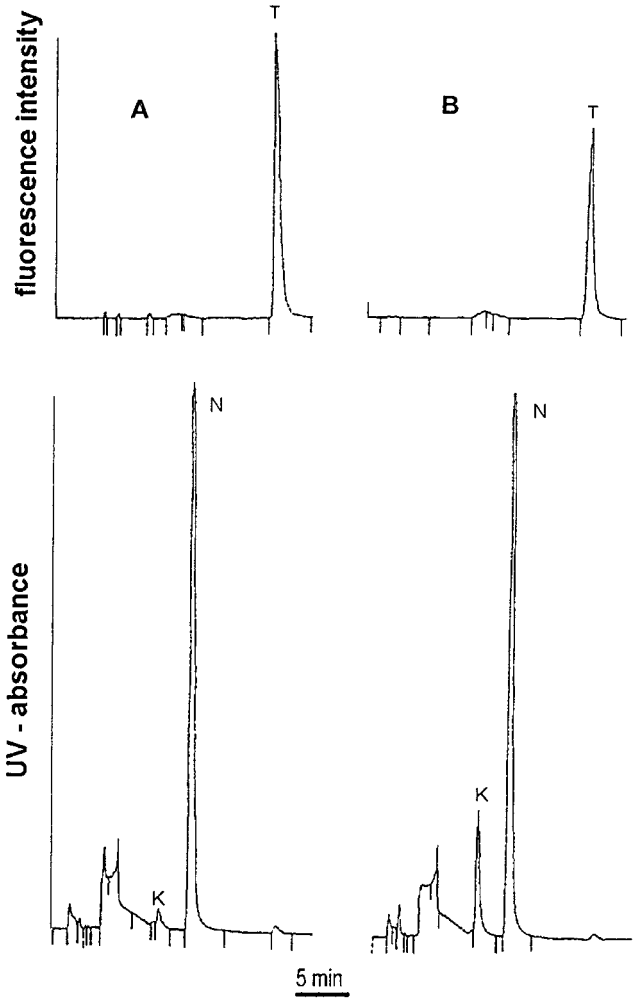

Fig. 1. Typical chromatogram of serum (*A*) and calibrator preparation (*B*).

T indicates the peak corresponding to tryptophan, *K* stands for kynurenine, and *N* stands for nitrotyrosine (actual concentrations of the external calibrator were 22.2 μmol/L tryptophan, 4.44 μmol/L kynurenine, and 44.4 μmol/L nitrotyrosine).

The introduced method provides a useful possibility to analyze tryptophan and kynurenine simultaneously in serum specimens, and hence enables direct insight into the tryptophan catabolism in a rapid and simple manner. As tryptophan and kynurenine concentrations vary over a comparatively wide range, the ratio of kynurenine to tryptophan may give reasonable evidence for an enhanced tryptophan degradation by the activity of IDO. Application of an albumin-based external calibrator in connection with internal nitrotyrosine secures analyses from possible influence of coprecipitation of tryptophan and (or) kynurenine when protein is removed by trichloroacetic acid.

Recent investigations provide evidence that nitrotyrosine is produced upon endogenous formation of nitric oxide radical *[15]*. Nitric oxide is synthesized by a variety of cells, especially when cytokine-inducible nitric oxide synthetase is activated. When nitric oxide reacts with superoxide anion produced by activated phagocytes, peroxynitrite is formed, which is a powerful oxidizing and nitrating species, producing, e.g., nitrotyrosine from tyrosine *[15]*. However, the concentration of the internal calibrator nitrotyrosine, which is applied in this method, exceeds the pathophysiological amounts by at least two orders of magnitude.

To conclude, the introduced method allows one to analyze the concentration for both the substrate and the first product of the IDO reaction, which is of interest in immunological research. The sample preparation is easy and rapid, and precision and accuracy are satisfactory. The method meets the requirements for routine analysis of tryptophan and kynurenine in serum and hence may provide a useful tool for clinical studies.

This work was financially supported by the Austrian Ministry of Science and Research, "Sektion Forschung".

References

1. Bender DA. The relative importance of dietary tryptophan and preformed nicotinic acid and nicotinamide as precursors of nicotinamide nucleotide coenzymes. In: Bender DA, Joseph MH, Kochen W, Steinhart HW, eds. Progress in tryptophan and serotonin research. Berlin: de Gruyter, 1986: 159–64.

2. Yoshida R, Imanishi J, Oku T, Kishida T, Hayaishi O. Induction of pulmonary indoleamine-2,3-dioxygenase by interferon. Proc Natl Acad Sci U S A 1981;78:129–32.
3. Hayaishi O, Yoshida R, Takikawa O, Yasui H. Indoleamine dioxygenase—a possible biological function. In: Schlossberger HG, Kochen W, Linzen B, Steinhart H, eds. Progress in tryptophan and serotonin research. Berlin: de Gruyter, 1984:33–42.
4. Yasui H, Takai K, Yoshida Y, Hayaishi O. Interferon enhances tryptophan metabolism by inducing pulmonary indoleamine-2,3-dioxygenase: its possible occurrence in cancer patients. Proc Natl Acad Sci U S A 1986;83: 6622–6.
5. Takikawa O, Yoshida,R, Kido R, Hayaishi O. Tryptophan degradation in mice initiated by indoleamine-2,3-dioxygenase. J Biol Chem 1986;261:3648–53.
6. Werner ER, Bitterlich G, Fuchs D, Hausen A, Reibnegger G, Szabo G, et al. Human macrophages degrade tryptophan upon induction by interferon gamma. Life Sci 1987;41:273–80.
7. Werner-Felmayer G, Werner ER, Fuchs D, Hausen A, Reibnegger G, Wachter H. Characteristics of interferon induced tryptophan metabolism in human cells *in vitro*. Biochim Biophys Acta 1989;1012:140–7.
8. Byrne GI, Lehmann LK, Kirschbaum JG, Borden EC, Lee CM, Brown RR. Induction of tryptophan degradation *in vitro* and *in vivo*: a gamma interferon stimulated activity. J Interferon Res 1986;6:389–96.
9. Werner ER, Fuchs D, Hausen A, Jaeger H, Reibnegger G, Werner-Felmayer G, et al. Tryptophan degradation in patients infected by human immunodeficiency virus. Biol Chem Hoppe Seyler 1988;369:337–40.
10. Fuchs D, Möller AA, Reibnegger G, Stöckle E, Werner ER, Wachter H. Decreased serum tryptophan in patients with HIV-1 infection correlates with increased serum neopterin and with neurologic/psychiatric symptoms. J Aquir Immune Defic Syndr 1990;3:873–6.
11. Rudzite V, Skards JI, Fuchs D, Reibnegger G, Wachter H. Serum kynurenine and neopterin concentrations in patients with cardiomyopathy. Immunol Lett 1992;32:125–30.
12. Denz H, Orth B, Weiss G, Herrmann R, Huber P, Wachter H, Fuchs D. Weight loss in patients with hematological neoplasias is associated with immune system stimulation. Clin Investig 1993;71:37–41.
13. Gasse T, Murr C, Meyersbach P, Schmutzhard E, Wachter H, Fuchs D. Neopterin production and tryptophan degradation in acute neuroborreliosis versus late Lyme encephalopathy. Eur J Clin Chem Clin Biochem 1994;32: 685–9.
14. Werner ER, Fuchs D, Hausen A, Reibnegger G, Wachter H. Simultaneous determination of neopterin and creatinine in serum with solid-phase extraction and on-line liquid chromatography. Clin Chem 1987;33:2028–33.
15. Kaur H, Halliwell B. Evidence for nitric oxide-mediated oxidative damage in chronic inflammation; nitrotyrosine in serum and synovial fluid from rheumatoid patients. FEBS Lett 1994;350:9–12.

Analytical Performance of Free PSA Immunoassays: Results from an Interlaboratory Survey, *Gian Carlo Zucchelli,[1]* Alessandro Pilo,[1] Maria Rosa Chiesa,[1] Richard Cohen,[2] and Ch. Albert Bizollon[2]* ([1]CNR, Inst. of Clin. Physiol., via Savi 8, 56100 Pisa, Italy; [2]Serv. de Radiopharmacie et de Radioanalyse, Univ. de Lyon, Lyon, France; *author for correspondence: fax 0039-50-553461, e-mail zucchell@po.ifc.pi.cnr.it)

Prostate-specific antigen (PSA) is a glycoprotein that is secreted by the prostate into the seminal fluid. Low concentrations of the protein are normally released into blood, but in prostate cancer (CAP) as well as in a high proportion of subjects with benign prostatic hyperplasia (BPH), serum PSA concentrations frequently increase above normal values (4 μg/L). Therefore, immunoassays measuring serum PSA concentration are routinely carried out to diagnose prostate diseases and to monitor progress of the disease and relapse of CAP after removal of the prostate *[1–3]*.

A few years ago, PSA was reported to be present in serum in three different forms. The predominant molecular form is complexed to α_1-antichymotrypsin, whereas a minor fraction circulates in a free noncomplexed form. These two forms are both measured by PSA assay. Only a very small proportion of PSA circulates bound to α_2-macroglobulin; this third form, however, is a nonimmunoreactive complex.

The free, noncomplexed form of PSA is reported to constitute a minute proportion of the serum PSA in patients with CAP, but to be significantly greater in subjects affected by BPH. On the basis of this observation the simultaneous measurement of total PSA (tPSA) and free PSA (fPSA) has been suggested. The computed ratio fPSA/tPSA is considered a useful tool to better discriminate between BPH subjects and CAP patients and therefore to improve the early diagnosis of CAP *[4]*.

Many immunoassays for fPSA measurement have been developed and are now commercially available. To evaluate the analytical performance of these assays, the international External Quality Assessment (EQA) program "Oncocheck" for tumor markers (AFP, CEA, CA 19–9, CA 15–3, CA 125, tPSA) organized by Service de Radiopharmacie et Radioanalyse, University of Lyon in cooperation with our Institute and Cis BioInternational has been extended to fPSA assay *[5, 6]*. About 300 laboratories participated in the 1996 EQA cycle, assaying tPSA; among these about 70 laboratories also assayed fPSA in control samples.

The most popular methods used by participants in the EQA for fPSA assay were IRMA Hybritech; IRMA Cis, Cis Biointernational; and ICMA Immulite, Diagnostic Products Corp. Each of these methods was used by about 20 laboratories.

Control samples were prepared by diluting a serum pool (tPSA concentration ~2000 μg/L) obtained from patients affected by CAP with normal human serum (tPSA concentration <0.5 μg/L); different dilutions were made to cover the entire assay range.

During the 1996 EQA cycle, 22 control samples (freeze-dried) were distributed and assayed; their average concentrations (consensus mean of all reported results) ranged from 1.99 to 28.1 μg/L for tPSA and from 0.15 to 2.07 μg/L for fPSA. The average between-laboratory agreement (or total variability, CV) of fPSA determinations was 28.0%. This variability was decomposed by ANOVA technique in the between-method and within-method components *[7, 8]*.

The within-method component (an estimate of the precision of the "average" method) was 21.8%, accounting for 60% of variability. This figure indicates that the methods for fPSA assays are affected by poor precision when compared with the within-method precision of tPSA (14.9%, computed from results of the same control samples).

The between-method component (which reflects the systematic differences in results produced by different methods) was 17.6%, accounting for the remaining 40% of the total variability. In fact, average fPSA results produced by the three most popular methods are consistently different from each other. This last observation is clearly