Homovanillic Acid in Plasma Determined by HPLC with Direct Injection of Plasma Filtrates

To the Editor:

The measurement of homovanillic acid (HVA) in plasma has been assessed in relation to several pathological processes, including tardive dystonias, epilepsy, schizophrenia, and other psychiatric illnesses. Various methods have been reported for the determination of HVA in plasma based on HPLC and electrochemical detection. Some of these methods are tedious and laborious (1–3); others require the use of an internal standard (4, 5) or more sophisticated instrumentation (6). We present here a modification of a previous method (1) in which the plasma is deproteinized by filtration and the filtrates are injected into an HPLC system.

From the previously reported chromatographic system (1) we modified the following parameters: the WISP 710B (Waters Associates, Milford, MA) automated injector was refrigerated with a module set at 4°C. Either an amperometric detector (Waters M460) set at 0.80 V or a coulometric detector (Coulochem II; ESA, Bedford, MA) could be used. The coulometric detector has a guard cell (Model 5021), installed after the column, set at 0.225 V and an analytical cell (Model 5011). The first electrode of the analytical cell was set at 0.2 V and the second one, an amperometric electrode that oxidizes between 75% and 85% of the product, at 0.4 V; the signal of the latter electrode was recorded. The chromatographic column was a Resolve C18, 300 × 3.9 mm, 5-μm particle size (Waters Associates). The mobile phase, precolumn, separation conditions, and quantitative analysis were as reported before (1).

For sample preparation we used centrifuge filters (Ultrafree MC low-binding cellulose, 30-kDa nominal molecular mass limit of exclusion), from Millipore, Bedford, MA. HVA (1 g/L) calibrator was prepared in mobile phase and stored in the refrigerator; daily dilutions in 1 mmol/L perchloric acid were made to give working concentrations of 0.16 and 0.08 mg/L. Plasma samples were thawed and centrifuged for 5 min at 4000g in a refrigerated centrifuge at 4°C, to avoid interference from plasma clots at the filter surface. (If fresh plasma is used, the centrifugation is not necessary.) Three 50-μL aliquots of plasma were pipetted into the centrifuge filters: 10 μL of 1 mmol/L perchloric acid was added to the first filter unit, and 10 μL of each HVA working solution (i.e., 0.8 and 1.6 ng of HVA, respectively) was added to the second and third filters. Samples were centrifuged for 30 min at 4000g at 4°C. Afterwards, 24 μL of each filtrate was injected into the chromatograph.

Chromatograms of a typical plasma sample are presented in Fig. 1. The plasma filtrates were stable at 4°C for at least 3 days—the CV of the peak height of an HVA calibrator solution being 2.8% and that of a plasma filtrate being 2.2% for 3 days’ injections. These variations with time could be explained by variability in detector response.

Detector responses were linear with concentration at least in the range of 0.08–1.44 ng of HVA injected. The complete procedure was also linear for the same amounts of HVA added to 50 μL of plasma. Analytical recovery of added HVA was 87.0% ± 3.8% (mean ± SD).

To assess reproducibility, we analyzed three pools of plasma in duplicate on 10 different days and obtained the following results (μg/L, mean ± SD): amperometric detector 4.3 ± 0.3, 7.1 ± 0.5, and 15.3 ± 1.1; coulometric detector 4.2 ± 0.3, 7.3 ± 0.5, and 15.6 ± 1.0. Voltammograms of the HVA calibrator and of the peak eluting at the same time as authentic HVA in plasma samples were identical in both detectors. Comparing the results of this method (γ) with those of our previous method (1) (r) gave a correlation of r = 0.976 (γ = 0.992 ± 0.427, range of concentrations of HVA 2–23 μg/L, n = 16). The correlation with a method based on gas chromatography–mass spectrometry (GC-MS, z) was also very good: r = 0.988 (γ = 1.067x− 0.509, range of concentrations of HVA 0–21 μg/L, n = 12). The above results support the identification of the peak that we measure as being authentic HVA.

The advent of amperometric and coulometric electrochemical detectors, which are highly selective and specific, has made possible the assessment of substances that, like HVA, exist in low concentrations in complex matrices such as plasma. A negative consequence of the high sensitivity of these detectors is interference from contaminants, e.g., the chemicals used in the sample clean-up procedure. As
we reported here, we overcome this problem by using the filters. We have not observed any late-eluting peaks that would interfere with the subsequent chromatogram nor any reduction in column life attributable to sample contamination. Moreover, the results we obtained with the amperometric detector are comparable with those of the coulometric detector.

We acknowledge Thomas B. Cooper, Nathan S. Kline Institute for Psychiatric Research in New York, for performing the analysis of plasma HVA by GC-MS, and thank the Servicio Vasco de Salud (Basque Health Service) for financial support.

References

Mercedes Zumárraga
Isabel Andía
Ricardo Dávila
María I. Zamalloa

Dept. de Invest. Neuroquím.
Serv. Vasco de Salud
Bº Arteaga, 107
48016 Zamalloa
Vizcaya, Spain

Author for correspondence.

Hydroxocobalamin and Sodium Thiosulfate Interferes Negatively with Measurement of Creatine Kinase Activity

To the Editor:

Hydroxocobalamin (OHCO) is able to combine with cyanide to form cyano-cobalamin (vitamin B₁₂). Because of this property, OHCO is used by clinicians with sodium thiosulfate as an antidote in cyanide poisoning (Cyanokit®; Laboratoires Anphar-Rolland, Lyon, France) (1). Administration of this drug in high concentrations (serum concentrations up to 500 μmol/L and 1.3 g/L for OHCO and sodium thiosulfate, respectively) leads to a red coloration of biological fluids, which can interfere with determination of some biochemical analyses such as bilirubin (2).

In one patient treated with Cyanokit, we observed different results in serum creatine kinase (EC 2.7.3.2; CK) activity assessed by Kodak Ektachem 700® (Eastman Kodak, Rochester, NY) and Hitachi 737® (Boehringer Mannheim, Mannheim, Germany) analyzers. The activities measured by these analyzers were 673 and 1016 U/L, respectively (reference range, 20–110), whereas CK activity in a previous sample (taken a few hours before the administration of Cyanokit) was 1225 U/L.

To investigate this discrepancy, we examined the interference of OHCO and sodium thiosulfate on CK activity measured by these two analyzers and by a manual assay performed according to the recommendations of the French Society of Clinical Biology (3). All three methods in the first step convert creatine phosphate and ADP to creatine and ATP by using N-acetylcysteine-reactivated CK. According to the manufacturer’s literature for the Kodak CK slide, ATP phosphorylates glycerol to α-glycerophosphate in the presence of glycerokinase; oxidation of α-glycerophosphate to dihydroxyacetone phosphate by α-glycerophosphate oxidase results in formation of hydrogen peroxide, which, in the presence of peroxidase, oxidizes a leuco dye precursor to form a dye, the rate of dye production being monitored by reflectance spectrophotometry at 670 nm. In the manual method (for which we used CK-GranuTest® reagents; Merck Clevon, Nogent sur Marne, France), ATP phosphorylates glucose to glucose 6-phosphate in the presence of hexokinase; oxidation of glucose 6-phosphate to gluconate 6-phosphate by glucose 6-phosphate dehydrogenase results in reduction of NADP⁺ to NADPH + H⁺, the absorbance of which is measured at 340 nm. The reaction is initiated by creatine phosphate after preincubation of serum with N-acetylcysteine. The Hitachi assay is an adaptation of the manual procedure and involves a unique reagent (Enzyline CK-NAC® Optimisé 100; Biomérieux, Marcy-l’Etoile, France), the reaction being initiated by the addition of serum.

Using the three methods, we compared CK activities in three pools of normolipemic nonicteric sera with high CK values (1300–1500 U/L), to which we had added increasing concentrations of OHCO (from 125 to 5000 μmol/L), sodium thiosulfate (from 0.3 to 13.3 g/L), and a mixture of both in the same proportions as supplied in Cyanokit. As shown in Fig. 1, OHCO interfered negatively in CK activity determinations by all three methods. In the presence of 500 μmol/L OHCO, the mean interference was −27%, −29%, and −21% in Kodak, Hitachi, and manual methods, respectively. The presence of sodium thiosulfate in high concentrations (up to 13.3 g/L) did not affect CK activity determined by methods measuring NADPH + H⁺

![Fig. 1. Interference from hydroxocobalamin (OHCO), sodium thiosulfate (S), or both (A) in the three methods: (A) Ektachem 700, (B) Hitachi 737, (C) manual method.](image-url)