Automated Enzymatic Analysis of Inulin
Anita L. Summerfield,¹ Glen L. Horta,²,³ Carl H. Smith,¹,² Timothy R. Wilhite,² and Michael Landt¹,²,⁴

We have developed an automated enzymatic assay for quantitation of inulin in plasma and urine that can be performed on the Cobas FARA II. In the assay, inulinase hydrolyzes inulin to fructose, and sorbitol dehydrogenase converts fructose to sorbitol with consumption of NADH, which is detected by spectrophotometry. The method incorporates a sample blank (inactivated inulinase) for each specimen to subtract contributions of endogenous fructose. Recovery of fructose or inulin was near 100%, with linearity to 300 mg/L. The enzymatic assay (y) agreed well with an anthrone comparison method (x) for analysis of inulin in both urine specimens (y = 1.00x - 138; S_{y|x} = 714) and plasma specimens (y = 1.00x - 3.5; S_{y|x} = 5.5). Glucose at 300 mg/L yielded an apparent inulin value of 1.3 mg/L in the enzymatic assay, but reacted at nearly 10% equivalency in the anthrone assay. Interferences from sorbitol, mannitol, and xylitol were negligible. CVs for day-to-day precision studies were 1–4%. The automated enzymatic assay of inulin is faster and avoids the use of caustic reagents required by the classical anthrone method.

Indexing Terms: glomerular filtration rate • renal function • fructose • inulin infusion • urine • diabetes

The most important measurement in assessing renal function is glomerular filtration rate (GFR) (1). Serum creatinine measurement is the most common method for estimating GFR because creatinine is endogenously produced, is fast and simple to measure, and provides a reasonable assessment of renal function in most medical situations. However, serum creatinine concentrations are relatively insensitive to GFR changes, because serum concentrations may not be abnormally increased until there is >50% loss of normal renal function (2, 3). Significant amounts of creatinine may be secreted by the renal tubule, and secretion increases with increasing renal failure, blunting the expected increase in serum creatinine concentrations and resulting in overestimation of GFR (4). Creatinine clearance improves estimation of the GFR over serum creatinine measurement alone, but requires timed urine collection, which adds additional sources of error (5). Although estimations of GFR based on creatinine clearance measure-

ments are sufficiently accurate for most clinical settings, alternative methods are needed for certain clinical situations in which more precise measurements of GFR are needed or endogenous creatinine production is reduced. Because the rate of creatinine production depends on muscle mass, plasma concentrations of creatinine may be very low in cachectic or elderly patients, or in infants and children. At low creatinine concentrations, interferences and analytical imprecision become much more significant (6).

A classic alternative method of measuring GFR is inulin clearance (for review, see 7). Inulin is a polysaccharide composed largely of fructose, with small amounts of glucose. From a metabolic standpoint, it is ideal for measuring GFR because it is freely filtered by the glomeruli and is not reabsorbed, secreted, or metabolically altered by the renal tubule. The principal disadvantage of this method is that inulin is not endogenously produced and thus requires intravenous administration with a long infusion time. Accurate determination of GFR requires several timed plasma and urine measurements. The most widely accepted laboratory method for quantitating inulin in plasma and urine is the anthrone reaction (8), which is based on the hydrolysis of inulin in hot concentrated sulfuric acid and condensation with anthrone to yield a green product that is read spectrophotometrically at 620 nm. The anthrone method is labor- and time-intensive, and other sugars such as glucose may interfere with the assay (8).

We have modified an enzymatic method, first proposed by Day and Workman (9), utilizing inulinase and sorbitol dehydrogenase to measure inulin. Our method is automated, fast, avoids the use of toxic reagents, and minimizes interference by glucose.

Materials and Methods
Specimens
Specimens for inulin analysis were obtained from pediatric patients at St. Louis Children's Hospital for routine physician-ordered renal assessment and in accordance with the requirements set for the study by the institutional review board. Briefly, a bolus loading dose of inulin was given via the antecubital vein and a continuous infusion (21 mL/h) was begun. The inulin concentration given to each patient was determined by estimates of body surface area and the degree of renal impairment. Blood specimens were collected from the antecubital vein of the arm opposite the infusion site immediately before bolus infusion and at 2, 3, and 4 h after the start of infusion. Urine samples were collected 2–3 h and 3–4 h after the start of infusion. All blood and urine specimens plus a sample of the infusate were analyzed.

Departments of ¹ Pathology and ² Pediatrics, Washington University School of Medicine at St. Louis Children's Hospital, St. Louis, MO 63110.
³ Current address: Department of Pathology, University of Alabama at Birmingham, 618 S. 18th St., WP P230, Birmingham, AL 35223.
⁴ Address correspondence to this author at: Department of Pediatrics, Washington University School of Medicine, One Children's Place, St. Louis, MO 63110. Fax 314-367-3765.

Received April 20, 1993; accepted July 7, 1993.
Reagents

Inulinase (Novozyme 230) (EC 3.2.1.7) was obtained as a liquid solution (24 000 U/L; 1 U = 1 μmol of fructose formed per minute) from Novo Nordisk Bioindustries (Danbury, CT). Sorbitol dehydrogenase (EC 1.1.1.14) was purchased in lyophilized form from Boehringer Mannheim Corp. (Indianapolis, IN). NADH in 2.0 mg preweighed vials and inulin from dahlias root were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and purchased from standard sources.

Methods

This two-step colorimetric assay was performed on the Cobas FARA II (Roche Diagnostic Systems, Montclair, NJ), with the settings listed in Table 1. The sample volume was 20 μL of heparinized plasma, 20 μL of urine [diluted 50-fold in 40 g/L bovine serum albumin (BSA)], or 20 μL of an infusate diluted 50–1000-fold with 40 g/L BSA. Properly diluted urine and infusate specimens could be analyzed in the same run as undiluted plasma. Specimens could be stored at 4 °C ≤ 1 week before analysis. The specimen was mixed on the analyzer with 100 μL of inulinase (diluted 100-fold with 10 mmol/L sodium phosphate, pH 5.0, immediately before use; final concentration 0.024 U/140 μL) and incubated for 10 min at 37 °C. Optimum pH for inulinase is near pH 5 (10, 11); a relatively weak buffer was chosen so that the pH could be readily altered by subsequent addition of a second enzyme solution. After digestion of the inulin to fructose, 80 μL of 80 000 U/L sorbitol dehydrogenase (final concentration 6.4 U/230 μL) with 800 mg/L NADH (final concentration 64 μg in 230 μL) in 0.25 mol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.6, was added. The mixture was incubated for 20 min, with the consumption of NADH (to reduce fructose to sorbitol) monitored by absorption readings at 340 nm.

Calibration was accomplished with 200 mg/L fructose in 40 g/L BSA. Blank values (endogenous fructose) were obtained for each specimen by assay with inulinase inactivated by incubation for 10 min in a boiling water bath. The inulin value was calculated by subtracting the value obtained for each specimen with inactivated inulinase. Inulinase stock remains stable for ≤ 6 months when stored at 4 °C. Sorbitol dehydrogenase may be stored for ≥ 2 weeks in solution at 4 °C.

Anthrone method. The determination of inulin in plasma and urine by the anthrone method was performed as described by White and Samson (8). Briefly, specimens were deproteinized by dilution (at least 15-fold) with 27 g/L trichloroacetic acid, and the precipitated protein was removed by centrifugation. Aliquots of each specimen (250 μL) were mixed with 2.5 mL of anthrone reagent (10 mmol/L in 700 mL/L sulfuric acid) and incubated in a 55 °C water bath for 10 min. After cooling (10 min in a cooling bath), the absorbance of each incubation was read in a spectrophotometer at 620 nm. Six calibrators containing 5–70 mg/L fructose were analyzed with each run.

Results

In the initial experiments we sought optimal conditions for hydrolysis of inulin and conversion of the resulting fructose to sorbitol. When inulinase activity (added in 80 μL) was varied from 2.4 to 2400 U/L, hydrolysis was complete over the linear range of the assay (see below) when the inulin activity exceeded 24 U/L. A standard inulinase concentration of 240 U/L (100-fold dilution of stock) was chosen to provide ample hydrolytic activity. The NADH concentration (357 μmol/L) was well above the Km of liver sorbitol dehydrogenase (20 μmol/L) (12) and was more than twice the stoichiometric amount needed for complete reaction over the linear range (see below) of the assay. Sorbitol dehydrogenase has a relatively high Km for fructose at pH 7.4, 350 mmol/L, which presents an analytical challenge (13). Because fructose concentrations generated during the assay were well below the Km, a high concentration of sorbitol dehydrogenase (6.4 U added) was needed to catalyze the reaction in a timely manner.

We compared the recovery of a known amount of fructose and inulin added to plasma when the calibrator was made in water with recovery calculated by means of
Fig. 1. Linearity of the enzymatic assay with known amounts of inulin added to plasma

Various amounts of a 5000 g/L stock inulin solution were added to aliquots of a plasma pool at constant plasma dilution of 100 mL/L. The dashed line represents \( x = y \)

The recovery of inulin in 40 g/L BSA. The recovery of fructose was low \( (y = 0.92x - 1.0) \) when the calibrator was prepared in water \( (y = \text{measured value}; x = \text{expected value}) \). When the calibrator was prepared in 40 g/L BSA, the recovery was \( -100\% (y = 0.98x + 3.5) \). The discrepancy was attributed to a matrix effect of protein in the enzymatic assay that was not eliminated by reducing the specimen volume or by extending the reaction time. Consequently, for all subsequent assays, we used a calibrator prepared in 40 g/L BSA, which effectively compensated for the matrix effect. The assay was linear for inulin concentrations up to 300 mg/L of inulin (Figure 1), with an apparent recovery of 99\% \( (y = 0.99x + 5) \). Because hydrolysis of 1 mg of inulin yields 1.11 mg of hexose, due to the addition of a water molecule to each sugar monomer, recovery could exceed 100\%; however, glucose is not measured by the assay (see below), and recovery of fructose from inulin hydrolysis will be reduced with increasing glucose content of the inulin. These opposing effects were apparently balanced in our inulin preparation, but other preparations may yield somewhat different recoveries of inulin. It is therefore important to analyze each inulin infusate to eliminate batch-to-batch variability in inulin glucose content.

Patients' specimens were analyzed by both the anthrone and the enzymatic assay. Values obtained from the anthrone assay \( (x) \) on plasma samples averaged slightly higher by a constant 3.5 mg/L than those from the enzymatic assay \( (y) \) \( (y = 1.00x - 3.5; S_{xy} = 5.5; \text{Figure 2, left}) \). The small constant bias between assays was not clinically significant. The correlation of urine and infusate analyses was excellent \( (y = 1.00x - 138; S_{xy} = 714; \text{Figure 2, right}) \). Because urine/infusate values are typically >3000 mg/L, the apparent constant bias of -138 mg/L was proportionally minor.

Interference by endogenous fructose and glucose was evaluated. Specimens from routine clinical inulin infusions (47 plasma and 21 urine, from fasting patients) were analyzed with the enzymatic method, omitting inulinase, to determine whether background fructose concentrations were significant. Background fructose concentrations in patients' plasma specimens ranged from 15 to 50 mg/L, but urine specimens contained negligible amounts. In another experiment, plasma specimens were collected from an individual just before and 1 h after ingestion of a soft drink containing fructose-based sweetener, and analyzed for apparent inulin (no inulin was infused). The anthrone analysis of the 1-h postingestion specimen yielded a value of 117 mg/L; subtraction of the zero-hour blank (15 mg/L) left an inulin content for the specimen of 102 mg/L. Enzymatic analysis of the 1-h postingestion specimen yielded 143 mg/L, but the inactivated inulinase value on the same specimen was also 143 mg/L; the net inulin was zero. Because these concentrations were significant in relation to the fructose generated by inulin hydrolysis, a blank determination (heat-inactivated inulinase) was

Fig. 2. Values obtained (left) on patients' plasma specimens or (right) on patients' urine and infusate specimens analyzed by the anthrone method compared with values obtained with the enzymatic method.

The data are corrected for 50-fold (or higher for infusion) dilution. The dashed line represents \( x = y \)
plasma was of results incorporated by interfered with discriminating substances. A mg/L, an inulin reference, enzyme described by work inulin was detected. When the same solution was analyzed by the enzymatic method, the inulin detected was only 1.3 mg/L. Interference studies performed with sorbitol, mannitol, and xylose showed that none of these substances was detected as inulin by either method over a range of 0–300 mg/L.

The precision of the assay was assessed by running daily quality-control plasma and urine samples for several weeks. The CV for plasma was 1.4% (x̄ = 160 mg/L, SD = 2.3 mg/L; n = 20) and for urine, 4.0% (x̄ = 2390 mg/L, SD = 96 mg/L; n = 25). The anthrone assay had a CV of 2.8% (x̄ = 645 mg/L, SD = 18 mg/L) for repeated analysis of a serum control.

Discussion

The anthrone method is currently the laboratory method most often used for quantifying inulin concentrations in serum and urine despite its inherent technical difficulty, labor intensiveness, and sensitivity to interfering substances (8, 14, 15). The enzymatic method described here improves the laboratory determination of inulin concentrations by eliminating the use of caustic reagents and by automating the method, which significantly reduces technical and labor constraints. The first enzyme in this assay hydrolyzes inulin into its individual fructose units (10, 11). Enzymatic hydrolysis avoids the hot acid hydrolysis used in the anthrone reaction. Sorbitol dehydrogenase, catalyzing the second reaction, specifically converts fructose to sorbitol, eliminating nonspecific reactions with other sugars, which may occur in the anthrone assay (12, 13, 16). The consumption of stoichiometric amounts of NADH by sorbitol dehydrogenase allows analysis of the reaction by conventional spectrophotometry in centrifugal and other standard clinical analyzers. Because aldose sugars such as glucose are not substrates for this enzyme (12), interference studies performed with glucose in this assay system show <1% reactivity.

Glucose interference in the anthrone method has limited the applicability of inulin clearance studies in diabetics, in whom plasma glucose concentrations may vary considerably during the several-hour infusion period. The present method, by nearly eliminating glucose interference, makes inulin clearance testing in diabetics more accurate. Recovery studies performed with known amounts of fructose and inulin showed that the enzymatic assay had excellent recovery and was linear up to a concentration of 300 mg/L, which covers the range of concentrations of inulin achieved in plasma during standard infusions. When patients' specimens were analyzed by both methods, we found good correlations between the enzymatic and anthrone methods for both plasma and urine. The precision of the enzymatic method was comparable with that of the anthrone method, with both assays yielding CVs of 1–4% in daily analysis of quality-control plasma and urine specimens.

Several recent publications have offered methods for inulin measurement designed to decrease interference by glucose and to speed analysis (17–19), but none is fully automated and all require more time to perform than the method described here. An HPLC method (17) has been developed that eliminates the potential interference of glucose and other noninulin chromophores in the anthrone method, but specimens must be analyzed singly on dedicated equipment. Enzymatic methods have been described that utilize inulinas for hydrolysis of inulin and convert the liberated fructose to glucose 6-phosphate for oxidation to gluconate 6-phosphate (18–20); these manual methods involve a cascade of several enzymes and are not easily automated. Glucose oxidase may be added before the hydrolysis step to oxidize endogenous glucose and eliminate glucose interference in subsequent enzymatic steps (18, 20). The overall time required for these two-step analyses exceeds 2 h. The method presented here was first proposed by Day and Workman (9) as a manual procedure, but was not evaluated for clinical use.

The physiological presence of fructose in plasma creates background interference for both the enzymatic and anthrone methods. The anthrone method requires a plasma specimen collected just before inulin infusion begins; the value of this specimen is subtracted as a blank from the values obtained for plasma specimens collected during infusion. This blanking mechanism assumes that the background presence of noninulin chromophores stays constant during the 3–4-h infusion, which may not be true for patients such as diabetics. Preinfusion ingestion of fructose- or sucrose-containing foods could also affect the blank value used in the anthrone method-based calculations; subsequent metabolism of the fructose over the subsequent several-hours-long infusion would decrease the background color development for subsequently drawn plasma specimens, and thereby underestimate the actual inulin concentration and artificially increase the calculated GFR. The enzymatic method, incorporating a true blank for each specimen, eliminates the potential for both overestimation and underestimation of inulin clearance in patients who are not fasting, which is an advantage for measurements in children and noncompliant patients.

References


Platelet Serotonin Content and Free and Total Plasma Tryptophan in Healthy Volunteers during 24 Hours

Nathalie Eyraud, Elisabeth Flachaire,2 Catherine Lestra, Martine Broyer, Radwan Zaidan,1 Bruno Claustrat,1 and Claude Quincy

The determination of platelet serotonin (5-HT) and plasma tryptophan concentrations is useful in the diagnosis, investigation of etiologies, and treatment of psychiatric disorders. To determine the usual circadian variations in platelet 5-HT and free and total tryptophan concentrations, we measured these variables during 24 h at 1-h intervals and every 30 min from 2000 to 0800 in seven clinically healthy young men with an HPLC method. No common circadian rhythm for platelet serotonin concentrations was observed in our subjects; however, there was a distinct rhythm for both free and total plasma tryptophan: Concentrations were maximal in the afternoon and minimal during the night.

Indexing Terms: circadian rhythms · psychiatry · chronobiology

Serotonin (5-HT) has been implicated in the coordination of mood, sleep, motor activity, thermoregulation, sexual activity, aggression, feeding, learning, and memory.5 5-HT is also implicated in major disorders such as depression, mania, schizophrenia, anxiety, pain, migraine, alcoholism, and even Alzheimer disease. Thus, determinations of concentrations of 5-HT and of its precursor and metabolites in plasma, cerebrospinal fluid, and postmortem tissue have been used in an attempt to understand the physiopathology of the serotonergic system and the mechanism of action of drugs that interfere with 5-HT metabolism. However, the use of 5-HT and tryptophan determinations in blood as a tool for clinical and pharmacological studies requires knowledge of the usual physiological circadian variations of 5-HT and its precursor. Currently, results from studies of the daily patterns of these variables are contradictory. The aim of this study was to determine the 5-HT and tryptophan profile over a 24-h period in clinically healthy subjects in controlled environmental conditions (diet, temperature, and activity).

Materials and Methods

Subjects and Blood-Sample Procedure

Seven clinically healthy young men, ages 23–32 years (mean ± SD, 27 ± 2.8 years), were studied between January and March 1992. All were students or members of the hospital staff and were nonsmokers. They had given their written informed consent; the study protocol was approved by the Local Ethics Committee. Medical examination revealed no illness at the time of the study, and no subject had required treatment with drugs for at least 1 month before the study. The subjects had no history of psychiatric disorders. They volunteered to spend 24 h (from 1200 to 1200) at the hospital in a special unit reserved for the experiment. They were submitted to a standardized schedule that consisted of balanced meals served at 1230, 1330, and 0800. They

Received June 30, 1992; accepted June 18, 1993.

CLINICAL CHEMISTRY, Vol. 39, No. 11, 1993 2337