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An Enzymic Assay for Uric Acid in Serum and Urine Compared with HPLC

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Summary: We evaluated a colorimetric method for the assay of uric acid in serum or urine, which utilises a *Trinder* chromogenic system modified by the inclusion of 2,4,6-tribromo-3-hydroxybenzoic acid for oxidative coupling to *p*-aminophenazone. Colour development (A_{max}: 512 nm) is complete within five minutes. Measurement of a sample blank is not needed. The procedure involves pre-incubation with ascorbic acid oxidase and detergent to eliminate interference by ascorbic acid and to abolish turbidity due to lipaemia; this pretreatment was effective up to 1.14 mmol/l ascorbate and up to at least 25 mmol/l triacylglycerol. Interference by icteric sera was insignificant up to about 170 µmol/l bilirubin. The method is linear up to at least 1428 µmol/l. In human serum and urine the procedure correlates well with HPLC and the uricase *p*-aminophenazone method on the SMAC analyser. Within-run and between-run imprecisions of the enzymic test were higher than for HPLC, but did not exceed 1.2% (CV) and 2.5% (CV), respectively.

Introduction

The concentration of uric acid in serum is the most important parameter for the diagnosis of gout. Of the methods developed (1–4) for the determination of this analyte, those utilizing the highly specific uricase[1]) reaction, with subsequent estimation of the H_2O_2 from the oxidation of uric acid, have gained widespread routine use.

Attempts to improve the detection of H_2O_2 led to the use of substituted phenols like 3,5-dichloro-2-hydroxybenzenesulphonic acid (5) and 2,4-dichlorophenol (6). By oxidative coupling with *p*-aminophenazone, these compounds give a quinoneimine dye, whose molar absorbance is 3 times greater than that of the original *Trinder* reaction (7).

The phenolic component in our uric acid *p*-aminophenazone reagent is 2,4,6-tribromo-3-hydroxybenzoic acid (fig. 1). The molar absorbance of its coupling product with *p*-aminophenazone exceeds that of the compounds used by *Fossati* et al. (5) and *Klose* and co-workers (6).

[1]) Enzymes:
Ascorbic acid oxidase (EC 1.10.3.3)
Peroxidase (EC 1.11.1.7)
Uricase (EC 1.7.3.3)

The present communication reports the imprecision, analytical range limits, interference by endogeneous serum constituents and accuracy of the uric acid *p*-aminophenazone test, and compares them with those of an HPLC method of established accuracy.

Materials and Methods

Analytical procedures

The Boehringer Mannheim uric acid *p*-aminophenazone test kit for mechanized analyses in serum, plasma and urine (Cat. No. 908 240, Boehringer Mannheim GmbH, FRG) was the subject of this evaluation study. Reagent 1 contains 0.1 mol/l potassium phosphate (pH 7.8); 6 g/l poly(ethyleneglycol-alkylether); 9.6 mmol/l sodium cholate; 24 mmol/l 2,4,6-tribromo-3-hydroxybenzoic acid; ≥ 5 kU/l ascorbate oxidase[1]).

The reaction is started by addition of reagent 2 [0.1 mol/l potassium phosphate, pH 7.8; 60 mmol/l potassium hexacyanoferrate (II); 0.12 mmol/l 4-aminoantipyrine; ≥ 1 kU/l peroxidase[1]); ≥ 0.5 kU/l uricase[1])]. The reaction sequence is depicted in figure 1.

The test was run on a Boehringer Mannheim/Hitachi 705 analysis system with instrument settings according to the manufacturer's recommendations:

Sample:	serum, urine (urine: diluted 1 : 5 with redistilled water)
Temperature:	25 °C
Sample volume:	10 µl
Volume of R1:	350 µl
Volume of R2:	70 µl
Measuring wavelength:	600 nm
Reference wavelength:	505 nm
Preincubation time:	5 min
Incubation time:	5 min

For comparison purposes, the Boehringer Mannheim uricase *p*-aminophenazone test (Cat. No. 157 104, Boehringer Mannheim GmbH, FRG) was used on a SMAC analyser as described by *Klose* et al. (6).

HPLC was a reverse phase procedure developed by *Greiling* et al. (8).

GC/MS measurements of uric acid concentrations were performed as published by *Siekmann* (9) in four human serum-based lyophilized sera.

All mechanized analyses with the uric acid *p*-aminophenazone reagent were calibrated with the "calibrator for automated systems" (Cat. No. 759 350, Lot No. 151 264, Boehringer Mannheim GmbH, FRG) which is based on lyophilized human serum. The calibration factor was determined by HPLC (10).

For purposes of quality control, we used four control sera (Precinorm® U, Cat. No. 171 735, Lot No. 152 620 and Lot No. 1-502; Precipath® U, Cat. No. 171 760, Lot Nos. 151 608 and 1-528, Boehringer Mannheim GmbH, FRG), an aqueous standard solution (Preciset® Uric Acid, Cat. No. 125 628, Boehringer Mannheim GmbH, FRG) and human serum-based lyophilized standards.

In order to prevent degradation of uric acid via urate anion free radical formation (11), the samples were stored in the dark until analysis.

Imprecision

Pool sera and control sera were analyzed with the uric acid *p*-aminophenazone test and the reference method at four different concentration levels. Within-series imprecision was derived from 20 measurements at each concentration level with fresh sera. Between-day imprecision was determined from measurements over 20 days with deep-frozen human sera and control sera.

Linearity and analytical range limits

A dilution series, prepared by mixing varying amounts of a uric acid-supplemented human serum and the same serum without added uric acid, was used to check the linearity. The lower limit of detection was calculated according to *Kaiser* (12), i.e. from the mean plus three S.D. of 20 measurements of the blank.

Accuracy

The accuracy of the new method was investigated as follows.

(A) Seventy fresh human sera and 40 urine samples covering a wide concentration range were assayed with the new uric acid *p*-aminophenazone test on the Hitachi 705, with the uricase *p*-aminophenazone method on the SMAC analyser, and with the HPLC method, and the results were compared.

(B) Four human serum-based lyophilized standards were analysed with the new method, with the reference method (GC/MS) and with the HPLC method, and the results were compared.

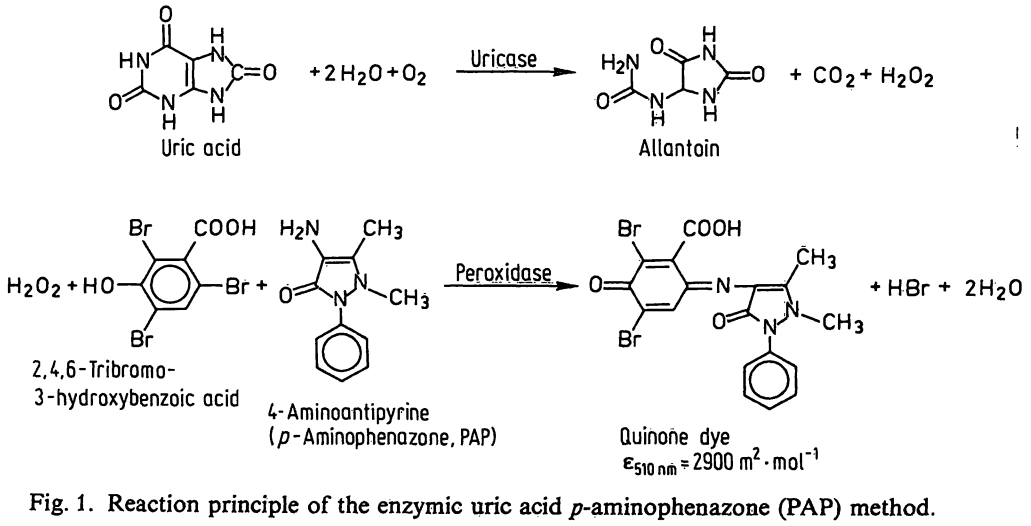

Fig. 1. Reaction principle of the enzymic uric acid *p*-aminophenazone (PAP) method.

(C) Using the new method, uric acid was measured in duplicate in a 384 µmol/l aqueous primary standard solution (SRM No. 913, National Bureau of Standards) which was used for standardization of the HPLC reference method.

(D) Using the new method, uric acid was measured in duplicate in a human serum-based reference material (SRM No. 909, National Bureau of Standards) with a certified value for uric acid which was also confirmed by the HPLC method used in the present study.

(E) The recovery of test-specific target values was measured in duplicate in control sera over a period of 20 days, using the new method. The test-specific target values were established with a reference standardization in three independent laboratories using HPLC (8). The data obtained by each laboratory for each of the respective sera in five independent runs and duplicate measurements were used for the assignment of target values.

Interference

Interference by haemoglobin was evaluated by adding increasing amounts of erythrocyte lysates to aliquots of pooled serum. The haemoglobin concentrations were quantified with a cyanmethaemoglobin method (Cat. No. 124729, Boehringer Mannheim GmbH, FRG).

We used two methods to examine the effect of bilirubin on the uric acid *p*-aminophenazone reagent: the new method was applied to a series of specimens with added crystalline bilirubin, and to icteric sera containing up to 461 µmol/l native bilirubin; the same samples were also analysed with the HPLC method. Bilirubin concentrations were measured with the DPD method (Cat. No. 398128, Boehringer Mannheim GmbH, FRG).

Possible interference by lipaemia was simulated by supplementing serum with Intralipid® (Pfrimmer & Co., FRG). A series of concentrations was prepared by mixing an aliquot of serum containing 10 g/l Intralipid® with an aliquot of the same Intralipid®-free serum. To compensate for the variable dilution resulting from the addition of lipids, we added proportional amounts of 0.154 mol/l NaCl solution.

A total of 34 pharmaceuticals (see tab. 1) was tested in vitro according to l.c. (13).

Potential interference by ascorbic acid was investigated with a series of concentrations prepared by mixing aliquots of an ascorbic acid-supplemented serum pool (5.68 mmol/l) with variable amounts of the same serum without ascorbic acid. For these studies we used deep-frozen human sera.

Statistics

Regression was analysed as described by *Passing & Bablok* (14).

Results

Imprecision

The CV values obtained with the uric acid *p*-aminophenazone test did not exceed 1.2% and 2.5% for within-run and between-run imprecision, respectively (tab. 2). The imprecision study conducted with HPLC as the reference method revealed a within-run variability of about 0.5% CV and between-run imprecision CVs in the range of 0.5% to 0.8% (tab. 3).

Tab. 1. Pharmaceuticals tested for possible interferences in vitro

Substances	Maximum concentration tested [mg/l]	"Therapeutic concentration" [mg/l]
Acetylsalicylic acid	1000	200
Allopurinol	300	30
Ampicillin	1000	200
Ascorbic acid	1000	30
Bezafibrate	100	10
Calcium dobesilate	200	100
Carbochromen	30	3
Chloramphenicol	200	20
Chlordiazepoxide	30	3
Furosemide	1750	10
Glibenclamide	1	0.1
Indometacine	100	20
Methaqualone	50	5
α-Methyldopa	100	6
Nicotinic acid	400	40
Nitrofurantoin	18	6
Noramidopyrine	200	20
Oxazepam	10	2
Oxyphenbutazone	240	20
Oxypurinol	300	30
Oxytetracycline	160	16
Paracetamol	200	20
Phenobarbitone	250	50
Phenprocumon	20	2
Phenazopyridine	25	2.5
Phenytoin	200	20
Probenecid	1000	200
Procaine	2	2
Pyridamol	100	30
Pyritinol	20	2
Quinidine	60	6
Sulphamethoxazole	600	80
Theophylline	200	20
Trimethoprim	18	2

Tab. 2. Imprecision of the enzymic uric acid *p*-aminophenazone method

Imprecision	Mean [µmol/l]	CV [%]
Within run (n = 20)	263	0.8
	317	1.2
	499	1.0
	554	0.4
Between run (n = 20)	222	2.0
	263	2.5
	339	2.5
	549	2.0

Tab. 3. Imprecision of uric acid determinations with HPLC

Imprecision	Mean [µmol/l]	CV [%]
Within run (n = 20)	266	0.5
	322	0.5
	513	0.5
	552	0.4
Between run (n = 20)	245	0.7
	357	0.8
	511	0.5
	592	0.8

Analytical range limits, linearity

The lower detection limit of the enzymic uric acid test, determined according to *Kaiser* (12), was found to be practically zero. This value was confirmed by measuring the dose-response relationship in a series of aqueous solutions at uric acid concentrations of zero to 60 µmol/l (data not shown).

The dynamic range of linearity of the enzymic test was assessed by using human serum containing varying amounts of added uric acid. Within the tested concentration range, which extended up to 1428 µmol/l, the method was found to be linear (fig. 2).

Interferences

The usual anticoagulant concentrations (100 g/l NaF, 10 g/l EDTA, 100 · 10^3 U/l heparin, 50 g/l citrate) do not influence the test results with the enzymic reagent.

Turbidity caused by lipaemia did not interfere in the enzymic uric acid test, as demonstrated by the failure of 10 g/l added Intralipid® to produce aberrant values.

Up to 186 µmol/l haemoglobin, haemolysis causes no noticeable interference in the new enzymic test.

Studies on interference by bilirubin were equivocal. Addition of commercial bilirubin to human serum led to a proportional decrease in the apparent uric acid concentration. When 427 µmol/l crystalline bilirubin was added to serum containing uric acid in the normal range, the resulting decrease in the apparent uric acid concentration was approximately 60%.

When we compared the uric acid recovery of the enzymic method with that of HPLC in native icteric sera, we also invariably observed decreased apparent uric acid concentrations. In this case, however decrease was markedly smaller and, furthermore, it depended on the particular serum sample. For example, with an icteric specimen containing 461 µmol/l bilirubin we found a 22% deviation from the value obtained with HPLC; on the other hand, other serum specimens with 137—171 µmol/l bilirubin, showed no significant interference.

Of the 34 drugs tested, only calcium dobesilate, α-methyldopa and noramidopyrine interfered with the present uric acid *p*-aminophenazone test at therapeutic concentrations. Calcium dobesilate at 100 mg/l, α-methyldopa at 100 mg/l and noramidopyrine at 20 mg/l caused apparent decreases in the uric acid concentration of about 50, 10 and 11%, respectively.

Ascorbic acid does not cause erroneously low results, until its concentration exceeds 1.14 mmol/l.

Accuracy

As shown in figure 3, recovery from control sera with the *p*-aminophenazone reagent is 4—10% lower than with GC/MS and HPLC.

The recovery of test-specific target values with the new test in four different control sera was found to vary from 99.5 to 102.6% (tab. 4).

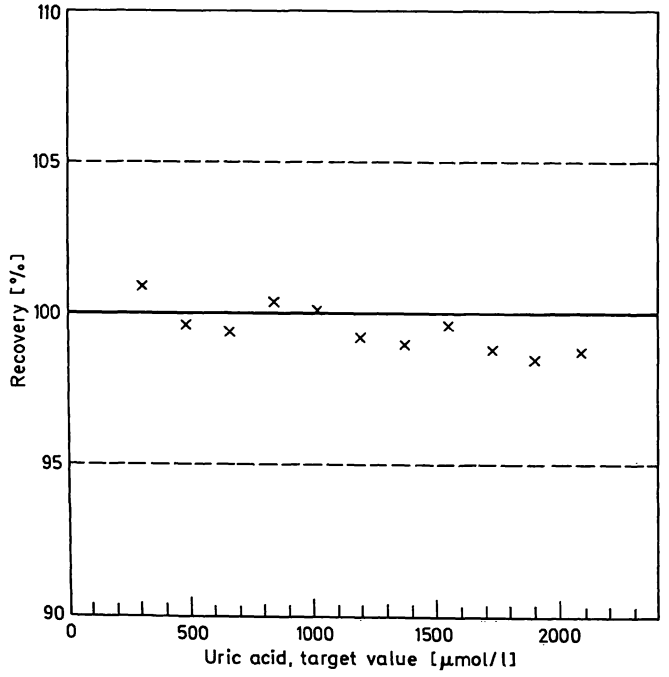

Fig. 2. Linearity of the enzymic uric acid *p*-aminophenazone (PAP) method.

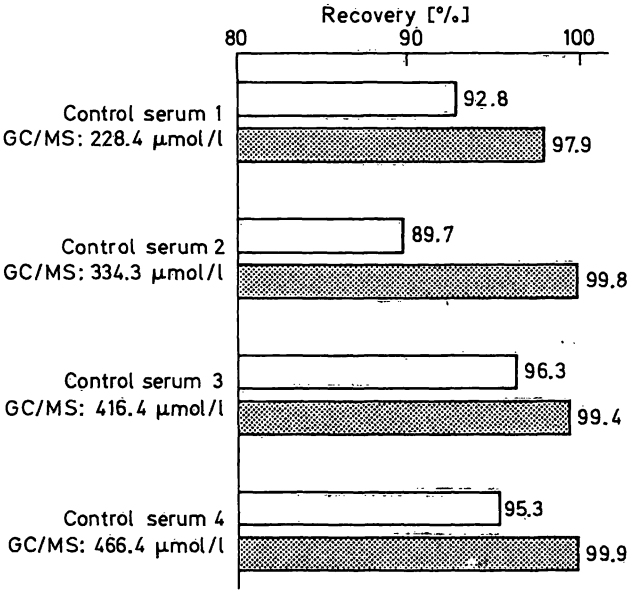

Fig. 3. Recovery of GC/MS values in control sera. White columns: Uric acid PAP; gray columns: HPLC

Tab. 4. Recovery of test-specific target values in control sera with the uric acid *p*-aminophenazone test

Target value [µmol/l]	Recovery ± S.D. [%]
246.3	102.6 ± 3.1
264.7	102.2 ± 2.8
536.6	101.4 ± 1.6
558.0	99.5 ± 3.7

Additionally, we examined the recovery with this colour test in the aqueous primary standard used for HPLC calibration and a human serum-based reference material. These measurements resulted in 100.3% and 99.9% recoveries of the respective target values (aqueous primary standard: 384 µmol/l; human serum-based control material: 483 µmol/l).

For method comparison, we analysed human serum and urine samples with the proposed method and HPLC. Furthermore, the uric acid *p*-aminophenazone test was compared with the uricase *p*-aminophenazone method on the SMAC analyser in human sera. We found the present test (y) to agree well with HPLC (x) in both human serum ($y = 0.30 + 0.97x$; $r = 0.995$) and urine ($y = 2.00 + 1.03x$; $r = 0.989$) (fig. 4, 5). A similar good agreement exists between the proposed method (y) and the uricase *p*-aminophenazone test (x) on the SMAC analyser ($y = -0.05 + 1.00x$; $r = 0.981$, fig. 6).

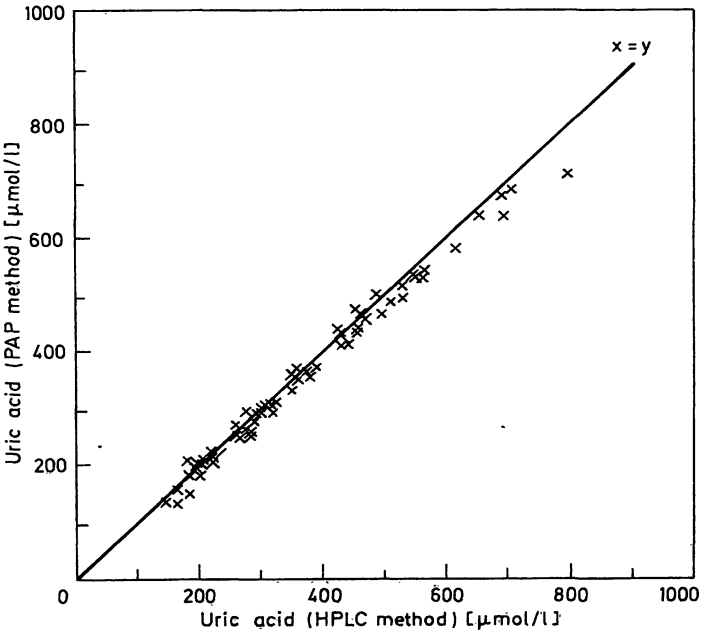

Fig. 4. Comparison of the uric acid *p*-aminophenazone (PAP) test (y) with HPLC (x), using human sera (n = 70, $y = 0.30 + 0.97x$, $r = 0.995$).

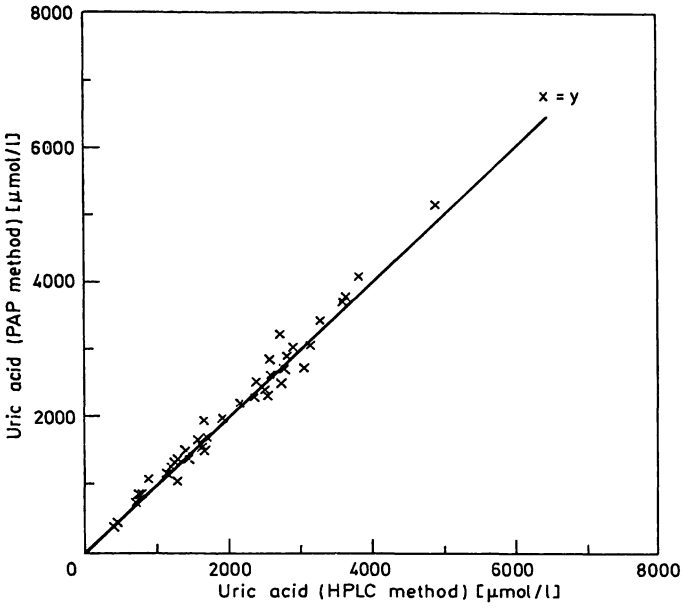

Fig. 5. Comparison of the uric acid *p*-aminophenazone (PAP) test (y) with HPLC (x), using urine samples (n = 40, $y = -2.00 + 1.03x$, $r = 0.989$).

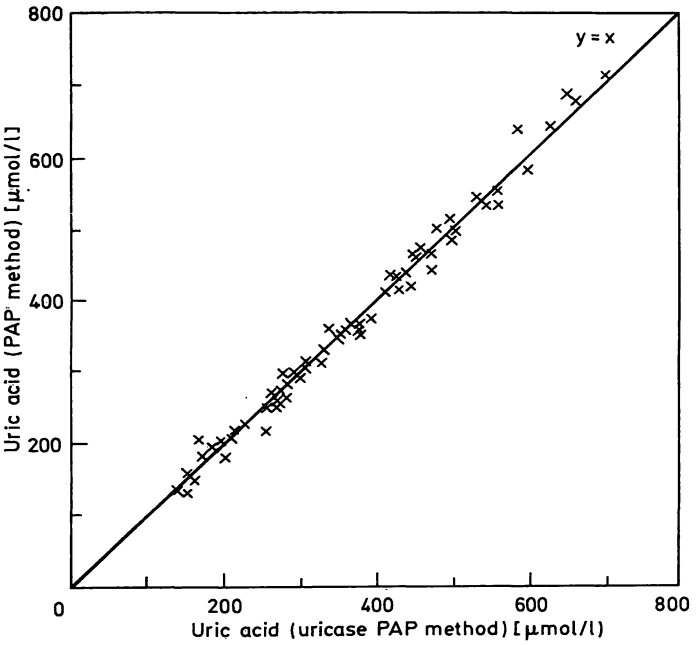

Fig. 6. Comparison of the uric acid *p*-aminophenazone (PAP) test (y) with the uricase *p*-aminophenazone (PAP) method on the SMAC analyser (x), using human sera (n = 70, $y = -4.54 + 1.01x$, $r = 0.990$).

Discussion

We evaluated an enzymic reagent for the determination of uric acid in serum and urine which utilizes a modified *Trinder* chromogenic system. The phenolic compound used couples oxidatively with *p*-aminophenazone, forming a quinone imine dye with improved molar absorbance. Hence, the sample/reagent ratio in our assay could be kept low, thus reducing possible interference by sample constituents to a minimum, while retaining satisfactory sensitivity and precision.

Optimization of component concentrations and assay pH ensured completion of colour development within 5 min at 25 °C or 37 °C, and a dynamic measuring range extending from zero concentration up to a level probably exceeding the highest level tested in this study which was 1428 µmol/l. Because of the short incubation times required for preincubation and maximum colour development, this assay can be conveniently adapted to mechanised systems which are capable of pipetting a start reagent.

In order to overcome the well known interference of ascorbic acid in *Trinder* chromogenic systems, a preincubation step has been included in this new assay during which any ascorbic acid is destroyed by ascorbate oxidase. The ascorbate degrading capacity in our assay is sufficient to eliminate ascorbate concentrations greater than 1 mmol/l within 5 min.

Interference by the turbidity of lipaemic sera was avoided in our test by addition of detergent to the preincubation solution, which proved to be effective at least up to a concentration of 10 g/l added lipid, being equivalent to about 28.6 mmol/l triacylglycerols (15).

Interference by bilirubin due to chemical and simple spectral effects has been reported for several peroxidase-coupled enzymic colour tests (16). When we measured uric acid concentrations with the new enzymic *p*-aminophenazone reagent in native icteric sera, however, we consistently observed significantly decreased recoveries of HPLC-defined analyte concentrations only when the bilirubin concentration was above 170 µmol/l. An explanation of this finding may be that the different bilirubin species contained in the individual pathological sera (17) have a different capacity for interference with the assay. Support of this view comes from data obtained recently with an enzymic creatinine *p*-aminophenazone method (18). In these studies, *Guder* et al. found variable relationship between assay interference and the bilirubin content of icteric sera, and significant disturbance was not detected up to 120 µmol/l bilirubin.

In the analysis of human serum and urine samples, as well as aqueous primary standard solution and human serum-based reference material with the enzymic *p*-aminophenazone method, we obtained results agreeing closely with those attained with HPLC, which has been proposed as reference method for uric acid determination (19). In control sera, on the other hand, the enzymic test yielded somewhat lower values compared with HPLC. Therefore, it will be essential to define specific target values in control material for the *p*-aminophenazone method.

In conclusion, the enzymic procedure evaluated in this study is considered to be a reliable method for routine uric acid determinations. Only samples with uncommonly high bilirubin levels, and specimens from patients receiving calcium dobesilate, α-methyldopa and noramidopyrine may give poor assay results by the proposed method. In such cases analyses should be run with a method known to be insensitive to these interferences.

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