

## Determination of uric acid in serum using isotachopheresis

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## DETERMINATION OF URIC ACID IN SERUM USING ISOTACHOPHORESIS

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### SUMMARY

An operational system is described for the isotachophoretic determination of uric acid in serum, making use of column coupling. The method has been compared with a standard enzymatic procedure. With the present technique small amounts of serum (ca. 3  $\mu$ l) can be applied without any pretreatment. Urate recovery was 99.0–100.5%. Under the non-physiological measuring conditions used, 12–28% of control serum uric acid was bound to macromolecules of molecular weight exceeding 25,000. The day-to-day variations of the isotachophoretic procedure were smaller than those of the enzymatic method, whereas standard deviations were comparable. The isotachophoretic procedure is less influenced by certain metabolites.

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### INTRODUCTION

Uric acid is the end-product of purine catabolism in man. An abnormal concentration of uric acid in body fluids may be indicative of a number of disturbances. An increased level of uric acid in serum (hyperuricemia) is seen in primary gout: although there is a normal excretion rate, uric acid is overproduced or it is underexcreted [1].

In secondary gout, hyperuricemia might result either from increased nucleic acid turn-over (for example, hematologic disorders, leukemia), or from decreased renal excretion of uric acid induced by drugs or dietary factors. Also in a number of genetic disorders, such as glycogen storage disease, Down's syn-

drome and psoriasis, is hyperuricemia a common feature [1, 2].

The methods currently used to assay uric acid in serum and urine are based on either chemical or enzymatic oxidation to allantoin. The enzymatic method seems to be the method of choice because of its sensitivity, accuracy and specificity [3, 4]. Analytical techniques such as high-performance liquid chromatography (HPLC) and isotachopheresis can be applied for determination in biological fluids of a series of metabolites, including uric acid. Methods using HPLC have been reported for serum urate [5], blood and urine [6]. Isotachopheresis has been employed in the analysis of nucleotides in muscle extracts [7] and urinary purines and pyrimidines, including uric acid [8]. Until now isotachopheresis has not been used for the rapid determination of serum uric acid.

Isotachopheresis is an electrophoretic separation method, taking advantage of the non-diluting phenomenon of the sample zone in the steady-state [9]. In this paper an isotachopheretic procedure is described for the quantification of uric acid in serum.

Unlike other available methods, such as the colorimetric [10] and the enzymatic [3, 4] methods, the determination of uric acid by HPLC and isotachopheresis is much less hampered by interfering substances, such as drugs and biological metabolites. In contrast to the HPLC procedure, where pretreatment of biological samples is often necessary (e.g. removal of proteins), the sample can mostly be applied directly in isotachopheresis. Moreover, the ratio of free to protein-bound urate can be determined conveniently using a simple ultrafiltration step.

## MATERIALS AND METHODS

### *Materials*

Uric acid (sodium salt), HCl (Titrisol<sup>®</sup>), tris(hydroxymethyl)aminomethane (Tris),  $\epsilon$ -aminocaproic acid (EACA) and morpholino-ethanesulfonic acid (MES), all analytical grade, were purchased from Merck (Darmstadt, G.F.R.). Hydroxyethylcellulose (HEC) was obtained from Polysciences (Warrington, PA, U.S.A.; Cat. No. 5568); a 0.5% (w/v) stock solution was purified by ion exchange. Uricase was purchased from Løvens Kemiska Fabrik (Ballerud, Denmark). Serum was prepared from venous blood after clotting (2 h at room temperature) and centrifugation for 10 min at 1000 g (4°C). The samples were stored at -20°C. Ultrafiltration CF 25 centriflow filters (mol. wt. cut-off 25,000) were purchased from Amicon (Oosterhout, The Netherlands).

### *Methodology*

In the present study isotachopheretic equipment was used in which two Teflon capillaries with different internal diameters were mounted [11, 12]. This set-up is of special interest for the analysis of biological samples. In such samples constituents are often present at a low concentration and the concentration of various compounds can differ by an order of magnitude. This is the case in serum, for example, where the chloride concentration can exceed the concentration of uric acid by a factor of up to 500. In such mixtures the analysis time required to obtain sufficient information on a given compound in-

creases when its concentration decreases. The present column coupling system [11, 12] alleviates this problem. Basically the equipment consists of two tubes with inside diameters of 0.8 mm (pre-separation tube) and 0.2 mm (separation tube). In the pre-separation tube a high electric current is permitted. At a well-defined distance from a "tell-tale" detector (conductivity type), mounted in the pre-separation compartment, a special construction (bifurcation) allows the separation tube to branch off from the pre-separation tube. The zones of interest can be easily selected via the "tell-tale" detector and separated from the "sample-train", migrating isotachophoretically in the pre-separation tube. In fact, the very efficient separation characteristic of isotachopheresis is applied for both sample pre-separation and final separation. A high sample load is permitted without a significant increase in analysis time. High ratios of concentrations between sample constituents are tolerated and different operational systems can be applied in one analysis. Moreover different electrophoretic principles (for example, isotachopheresis and zone electrophoresis) can be applied [9].

The enzymatic determination of serum uric acid was performed in the laboratory of the Department of Neurology (University Hospital, Nijmegen) with an ABA 100 bichromatic analyser (Abbott). The determination of uric acid is based on the successive action of three purified enzymes which are added to the reaction mixture: uricase, catalase and aldehyde dehydrogenase [4]. The formation of NADPH from NADP<sup>+</sup> in the latter reaction (measured at both 340 and 380 nm) is used for the quantification of uric acid. Sera containing known concentrations of uric acid were used as standards.

## RESULTS

The operational conditions for the isotachopheretic determination of uric acid are listed in Table I. The leading electrolyte (pH 5.0) contains Cl<sup>-</sup> as the leading ion; the terminating electrolyte (pH 6.5) consists of morpholino-ethanesulfonic acid and Tris. The analysis time with the column coupling system is 12 min.

Physiological uric acid concentrations normally range between 0.15 and 0.40 mM. In Fig. 1 the calibration curve for uric acid in this range is shown. As can be expected in isotachopheresis there is a linear relationship between the uric acid zone length and the amount of uric acid injected.

TABLE I  
OPERATIONAL SYSTEM FOR THE ISOTACHOPHORETIC DETERMINATION OF URIC ACID

	Electrolyte	
	Leading	Terminating
Anion	Chloride	MES
Concentration ( <i>M</i> )	0.01	0.005
Counter-ion	EACA	Tris
pH	5.00	6.5
Additive	0.25% HEC	
Driving current (A)	20×10 <sup>-6</sup>	

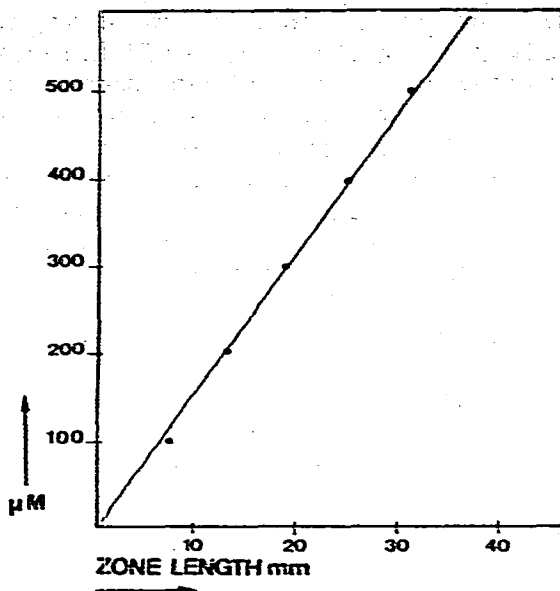


Fig. 1. Calibration curve for uric acid. Operational conditions, see Table I. Injected volume: 3  $\mu$ l.

When standard amounts of uric acid were added to serum, extensively dialysed against 0.9% NaCl, the isotachophoretic analyses yielded recoveries of 99.0–100.5% (data not shown). The uric acid zone was eliminated after treatment of the serum with purified uricase. In addition, over-spiking confirmed the identity of the uric acid zone.

In order to estimate the amount of uric acid bound to serum proteins under our experimental conditions, the recovery from ultrafiltered and non-ultrafiltered samples was compared. When undialysed pooled serum from several healthy controls was passed through an Amicon CF 25 filter (mol. wt. cut-off 25,000), 85.1% of the total serum uric acid was recovered in the ultrafiltrate, indicating that in this sample approximately 15% was bound to proteins with a molecular weight exceeding 25,000. The lower amount of uric acid in the ultrafiltrate as compared to non-filtered samples was not due to the CF 25 filter; when a standard solution of uric acid (474  $\mu$ M in water) was passed through it, the recovery was 99.4%. Also the effect of high pH on the binding of urate to serum proteins was studied. The pH of normal serum samples (pH 7.2–7.4) was adjusted with NaOH to pH 10.0 and after ultrafiltration the urate binding turned out to decrease to approximately 7%.

Some serum samples showed turbidity, as judged from visual inspection. Those samples were rapidly passed through a Millipore filter (Millex<sup>R</sup>, 0.22  $\mu$ m). This did not affect the recoveries.

Serum samples from six healthy controls were assayed for uric acid using the isotachophoretic method and the enzymatic method. The samples were used either directly or after ultrafiltration. The serum uric acid values obtained with both methods showed an acceptable correlation (Table II): correlation coefficient 0.98. Twelve to twenty-eight per cent of the uric acid seemed to be associated with macromolecules of molecular weight exceeding 25,000. The day-to-

TABLE II

## ISOTACHOPHORETIC AND ENZYMATIC DETERMINATION OF URIC ACID IN SERA FROM SIX HEALTHY CONTROLS

No	Isotachophoresis			Enzymatic method		
	NF* ( $\mu M$ )	UF** ( $\mu M$ )	Bound*** (%)	NF* ( $\mu M$ )	UF** ( $\mu M$ )	Bound*** (%)
1	374	329	12	390	303	22
2	392	282	28	383	283	26
3	294	224	24	292	233	20
4	483	415	14	483	400	17
5	361	298	17	375	317	15
6	463	385	17	498	365	27

\*NF = not ultrafiltered.

\*\*UF = ultrafiltered (CF 25).

\*\*\*Bound = the percentage of uric acid removed by ultrafiltration.

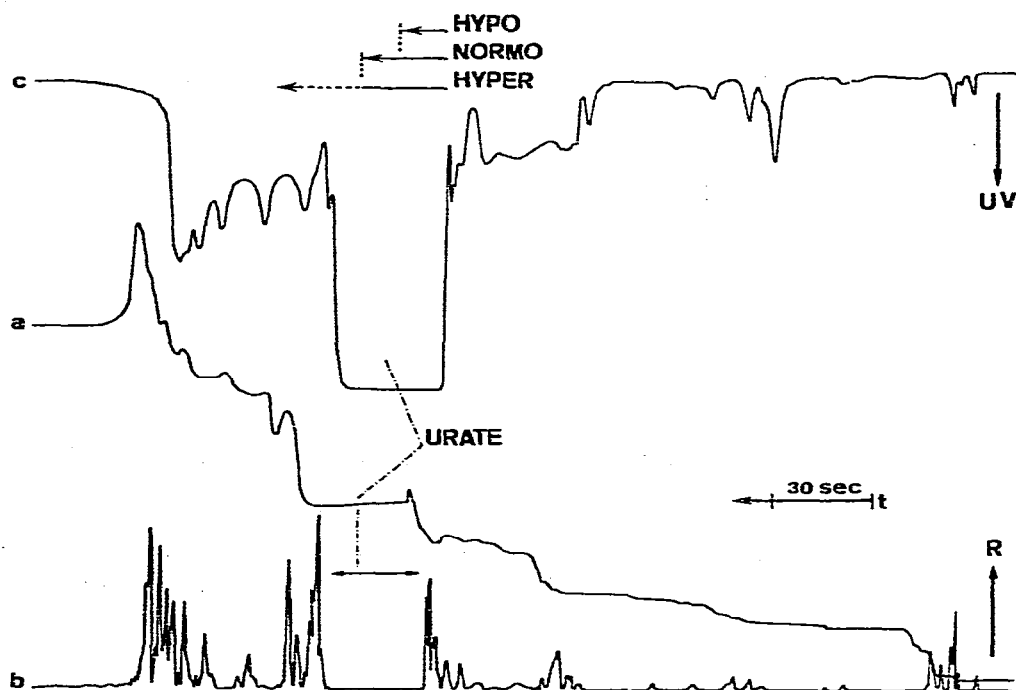


Fig. 2. Isotachophoretic separation of a hyperuricemic serum. The horizontal arrows give the uric acid zone lengths of hypo-, normo- and hyperuricemic sera under standardised operational conditions (Table I). a: conductivity signal, R = increasing resistance. b: differential signal of the conductivity signal. c: UV signal, transmission at 280 nm.

day variation for a repeatedly tested sample was ca. 2% with the isotachophoretic method and ca. 10% with the enzymatic method.

The uric acid zone length is directly representative of the serum concentration under the present operational conditions. In hypo-uricemic sera the zone length will be less than 13.5 mm. Normo- and hyperuricemic sera will give zones of up to 25 mm and longer than 25 mm, respectively (Figs. 1 and 2).

TABLE III

## THE EFFECT OF HOMOGENTISIC ACID ON URIC ACID DETERMINATION BY ISOTACHOPHORESIS AND THE ENZYMATIC METHOD

Addition	Isotachophoresis ( $\mu M$ )	Enzymatic method ( $\mu M$ )
None	348	348
0.5 g/l homogentisic acid	348	364
5.0 g/l homogentisic acid	346	676

TABLE IV

## SERUM URIC ACID CONCENTRATIONS IN HYPERURICEMIC PATIENTS RECEIVING VARIOUS DRUGS

Diagnosis	Medication	Uric acid ( $\mu M$ )	
		Enzymatic method	Isotachophoresis
Gout (male, age 63)	Zyloric	286	299
Rheumatoid arthritis with hyperuricemia (female, age 68)	Hygroton, Selokene, Penicillinamide, Indocid Seresta	620	647
Rheumatoid arthritis with hyperuricemia (female, age 44)	Baktrimel, Primmison, Torecan	356	366

Several metabolites and drugs can interfere with the enzymatic determination of uric acid. An example is homogentisic acid, a compound which occurs in increased quantities in urine of patients with alkaptonuria, an inborn error of amino acid metabolism [13, 14]. When unphysiologically high amounts of homogentisic acid were added to samples, no effect on the isotachophoretic determinations was seen. However, with the enzymatic method higher values than those actually present were recorded (Table III).

Serum uric acid values in three initially hyperuricemic rheumatologic patients, who were treated with a number of drugs, were in close agreement when determined by both procedures (Table IV). None of the drugs seemed to interfere with the uric acid determination according to both methods.

## DISCUSSION

The present isotachophoretic method for the determination of uric acid levels in serum is quantitative, reliable and reproducible (Fig. 1). In contrast to the general practice in HPLC [6] there is no need for deproteinisation: the samples can be applied directly. However, also a HPLC system without deproteinisation has been described [5]. It should be pointed out here, that in HPLC it is sometimes difficult to predict the retention behaviour of a given compound. In isotachophoresis this is more predictable on the basis of the  $pK_a$  values and mobilities of the compounds under study. An additional advantage of isotachophoresis over HPLC is the flexibility of the system: no column packing and equilibration is necessary if rapid switching from one electrolyte

system to another is needed when different conditions have to be tested. Once an electrolyte system has been chosen the isotachophoretic analyses can be done with very low day-to-day variations (< 2%). This value is lower than that obtained with the enzymatic method with a common day-to-day variation of 5–6% [15]. At the present time the isotachophoretic serum uric acid determination is more accurate than the enzymatic method, although the latter is faster when automated.

The physiological significance of the binding of urate to plasma proteins is still disputed. Reversible interactions between urate and serum albumin, low-density- $\beta$ -lipoprotein,  $\beta_2$ -macroglobulins and  $\alpha_1, \alpha_2$ -globulin have been reported [16, 17].

Percentages of 20–40% of bound urate have been described under different conditions of temperature, ionic strength, buffers, etc. [16, 18, 19]. Our values agree with these data (Table II). Reduced binding capacity of plasma proteins might lead to higher levels of free uric acid; in patients with gout such a decreased binding capacity has been reported [16, 18]. It has also been shown that several drugs, such as salicylates, phenylbutazone and probenecid, reduce urate binding in vitro [20]. It should be stressed, however, that all of these studies, including our own, were done under non-physiological conditions. Therefore, no conclusion can be drawn regarding the physiological significance, especially because in vivo measurements have shown that at 37°C the percentage of urate bound is small [21].

Homogentisic acid is a metabolite which occurs in increased quantities in the urine of alkaptonuric patients. It interferes with the enzymatic uric acid determination at 340 nm by causing lower values than those actually present to be recorded [4]. No effect was seen with isotachopheresis in the presence of homogentisic acid, whereas increased levels were read with the enzymatic procedure carried out with the bichromatic (380 and 340 nm) analyser ABA-100 (Table III). No attempts were made to elucidate this experimentally, but the differences might be attributed to the use of a bichromatic analyser in the present study, in contrast to a monochromatic determination at 340 nm only [4].

A number of drugs used in the treatment of three initially hyperuricemic patients did not interfere with any of the methods employed: the uric acid values obtained were comparable (Table IV).

The usefulness of isotachopheresis in screening for inborn errors of purine and pyrimidine metabolism by analysing urinary bases and nucleosides has already been demonstrated [8]. We have recently developed operational systems for the analysis of purine and pyrimidine nucleosides and bases in serum [22]. For both experimental and clinical purposes an alternative analytical approach is opened up, such as for the pharmacokinetic analysis of drug metabolism, or in tracing the consequences of metabolic disturbances using body fluids and cell lysates.

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