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ISOTACHOPHORESIS OF URINARY PURINES AND PYRIMIDINES THE USE OF SPACERS AND ENZYMES FOR IDENTIFICATION

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SUMMARY

An isotachophoretic system is described for the separation and identification of urinary purine and pyrimidine bases and nucleosides. For a better discrimination and interpretation of the UV profiles, well-defined non-UV-absorbing substances were introduced as spacers. Treatment of urine samples with purified enzymes before analysis resulted in specific shifts in the metabolite profiles, providing a sensitive and specific means of identifying a number of metabolites.

With an injected volume of 3 μ l (untreated urine diluted 1:5) the present method allows reproducible separations within 20 min of at least twenty different nucleosides and bases.

INTRODUCTION

Considerable progress has been made in the understanding of pathophysiological mechanisms, using analytical techniques that enable the simultaneous identification of metabolites participating in the same metabolic pathway. High-performance liquid chromatography (HPLC) especially has created new possibilities for, for example, the study of inborn errors of purine and pyrimidine metabolism and the pharmacokinetic analysis of purine and pyrimidine drug metabolism (for example, in cancer chemotherapy) [1]. Isotachophoresis

is another analytical technique that is suitable for monitoring metabolic intermediates [2]. It has been used for screening of purines and pyrimidines in urine and serum [3, 4].

During isotachophoretic separation, the ions (for example, metabolites) are separated in a buffered system according to differences in net mobility. The separated ions form zones, which move consecutively as a train at equal speed, with sharp boundaries between them [2]. In the case of purines and pyrimidines the zones are generally monitored with UV light at 254 nm and/or 280 nm [2-4].

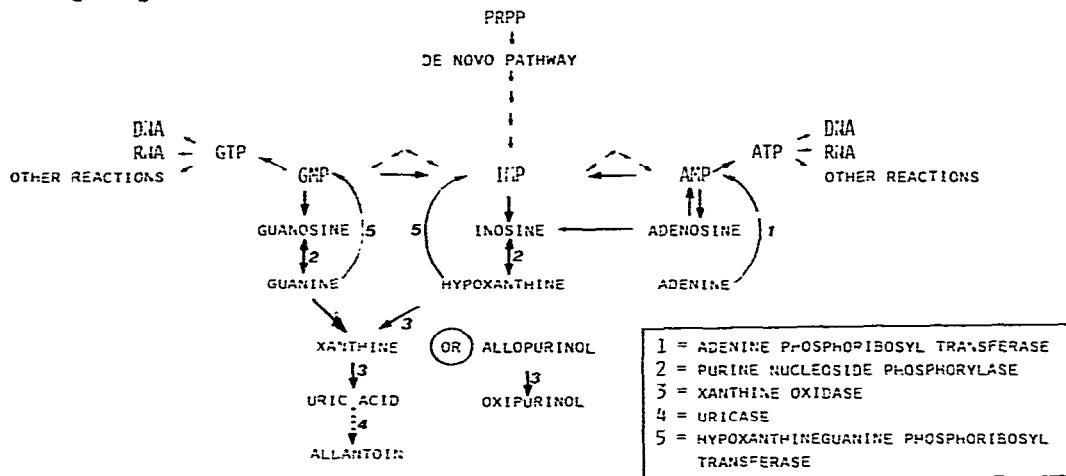


Fig. 1. Simplified scheme of purine metabolism in man.

Problems with the identification and quantification of metabolites may sometimes arise when a given compound forms an extremely small zone or when adjacent zones exhibit similar UV-absorption characteristics. The latter problem can be solved by using conductimetric detection (see Fig. 5 in ref. 5). Another solution might be provided by the use of non-UV-absorbing ionic spacers, which form discriminating zones between UV-absorbing compounds. Preincubation of a sample with a purified enzyme that acts specifically on a compound, might provide another solution. Disappearance of a certain metabolite will be accompanied by the formation of a new metabolite (product of the enzymatic reaction), which in some cases can be identified in the same isotachopherogram.

The purpose of this paper is to communicate the analysis of urinary purine and pyrimidine bases and nucleosides using a system of spacers and enzymes. A number of metabolites and enzymatic reactions relevant to the present study are depicted in Fig. 1.

MATERIALS AND METHODS

Equipment

The analyses were performed with an LKB 2127 Tachophor equipped with a 43-cm PTFE capillary tube (I.D. 0.5 mm) and thermostated at 20°C. The separation was monitored with a UV detector at 254 nm.

Electrolyte system

The operation system, as used in this study, is given in Table I.

TABLE I

OPERATIONAL SYSTEM FOR THE SEPARATION OF PURINE AND PYRIMIDINE METABOLITES BY ISOTACHOPHORESIS

	Leading electrolyte*	Terminating electrolyte*
Anion	Chloride	β -Alanine/ OH^-
Concentration	0.005 M	0.02 M
Counter-ion	Ammediol ⁺	H^+/Ba^+
pH	8.55 \pm 0.02	10.4-10.5
Additive	0.3% hydroxyethylcellulose	None

* β -Alanine, ammediol (2-amino-2-methyl-1,3-propanediol), HCl and Ba(OH)₂ were all purchased from Merck and were of analytical grade. The first two chemicals were further purified by recrystallization with methanol. Hydroxyethylcellulose was purchased from Polysciences Inc. (Warrington, PA, U.S.A.) and was purified by ion exchange using mixed-bed exchanger No. V (Merck).

Spacers

For better discrimination and interpretation a study was made to test non-UV-absorbing compounds as spacers. Table II shows, on the left, a list of purines and pyrimidines, according to their net mobility, which can be separated by the system described in Table I. On the right-hand side Table II presents a list of non-UV-absorbing compounds. Those that have net mobilities equal to certain purines and pyrimidines are indicated on the same lines. Those compounds that have intermediate net mobilities are listed between the relevant purines and pyrimidines.

Enzymatic identification

The following enzymes were used: PNP (purine-nucleoside phosphorylase EC 2.4.2.1, from calf-spleen) was purchased from Boehringer (Mannheim, G.F.R.); XO (xanthine oxidase EC 1.2.3.2, from buttermilk) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); uricase (EC 1.7.3.3) was purchased from Løvens Kemische Fabrik (Malmö, Sweden); A-PRT (adenine phosphoribosyltransferase EC 2.4.2.7) was purified according to the method of Hershey and Taylor [6].

As co-substrates for the PNP and A-PRT reactions ribose-1-phosphate (Boehringer) and phosphoribosylpyrophosphate (Sigma), respectively, were used.

For enzymatic identification 100 μl of diluted (1:5) urine were incubated for various periods of time at 37°C. For the PNP incubation 3 μl of enzyme-solution were added in the presence of 0.5 mM ribose-1-phosphate; incubation time was 2.75 h. For the A-PRT reaction 3 μl of purified enzyme fraction were added in the presence of 1 mM phosphoribosylpyrophosphate; incubation time was 2 h. For the XO reaction 3 μl of enzyme solution were used; incubation time was 3 h. For the uricase reaction 6 μl of enzyme solution were added; incubation time was 22 h.

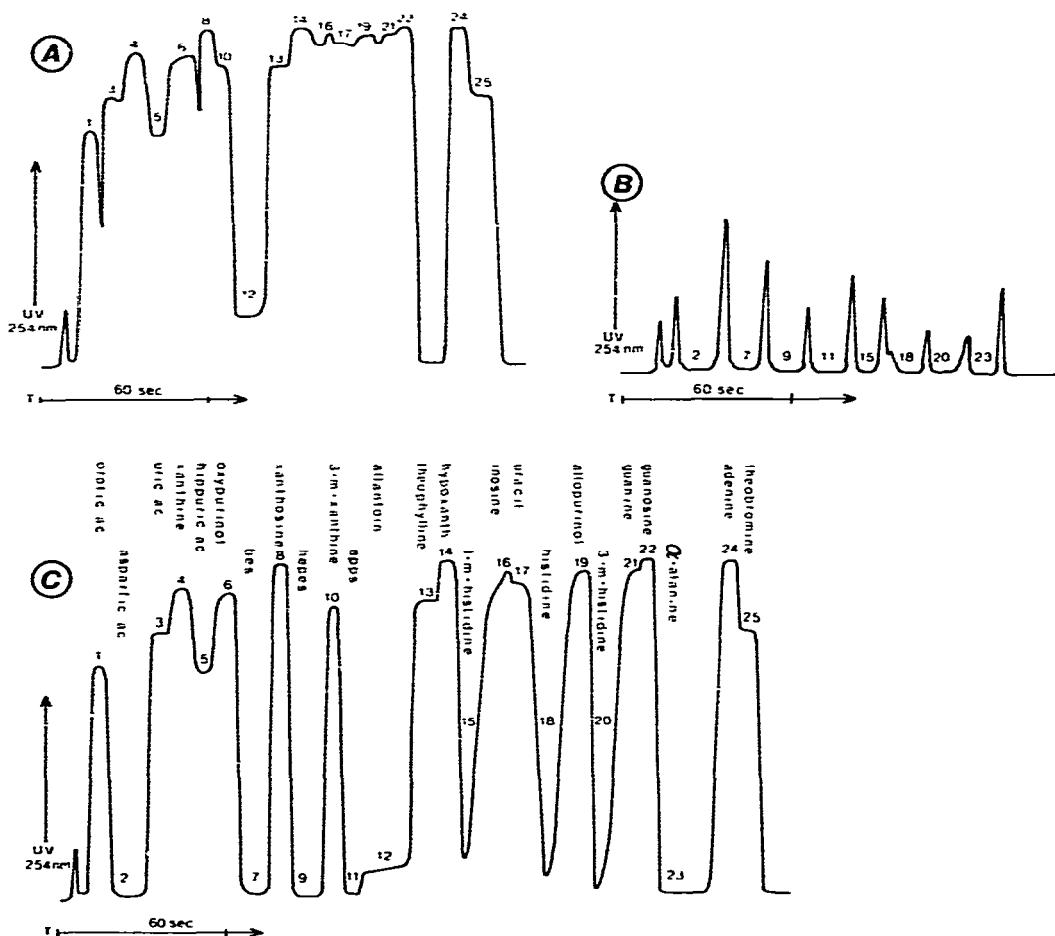


Fig. 2. The isotachophoretic analysis of 17 UV-absorbing compounds (A), 8 non-UV-absorbing spacers (B) and a mixture of the two types of compounds (C). 1 = orotic acid; 2 = aspartic acid; 3 = uric acid; 4 = xanthine; 5 = hippuric acid; 6 = oxypurinol; 7 = BES; 8 = xanthosine; 9 = HEPES; 10 = 3-methylxanthine; 11 = EPPS; 12 = allantoin; 13 = theophylline; 14 = hypoxanthine; 15 = 1-methylhistidine; 16 = inosine; 17 = uracil; 18 = histidine; 19 = allopurinol; 20 = 3-methylhistidine; 21 = guanine; 22 = guanosine; 23 = α -alanine; 24 = adenine; 25 = theobromine. The zone-lengths represent approx. 1.5 nmol of each substance. For abbreviations see Table II.

RESULTS

An operational electrolyte system that gives reproducible separations of urinary nucleosides and bases is given in Table I, the leading ion being Cl^- , the counter-ion ammediol and the terminating ion β -alanine. Analysis time is approximately 20 min. In order to speed up the analysis, during the initial separation a driving current of $180 \mu\text{A}$ was applied; during the last 15 min the current was $40 \mu\text{A}$. The injected volume of the diluted* (1:5) urine sample was

*Dilution guarantees that certain substances, for example urate and oxalate, which might be present as precipitates will redissolve.

TABLE II

PURINES AND PYRIMIDINES AND A NUMBER OF NON-UV-ABSORBING COMPOUNDS, LISTED ACCORDING TO THEIR RELATIVE NET MOBILITIES

Purines and pyrimidines	Compounds* useful as spacers (non-UV-absorbing)	Non-UV-absorbing compounds**
Orotic acid (1)**	<i>Aspartic acid</i> (1)	Capronic acid/isobutyric acid
Uric acid (1)		Caproic acid/glutamic acid/ACES
Xanthine (1)	<i>MES</i> (2)	
Hippuric acid (1)		MOPS
Oxypurinol (3)		
Orotidine (1)		
Xanthosine (1)	<i>BES</i> (1)	Cystine/TES
3-Methylxanthine (1)	<i>HEPES</i> (1)	TRICINE
Allantoin (1)	<i>EPPS</i> (1)	TAPS
Theophylline (1)		
Hypoxanthine (2)	<i>1-Methylhistidine</i> (1)	Asparagine
Inosine (2)		Serine
d-Inosine (1)		
Uracil (1)	<i>Histidine</i> (2)	Methionine/glutamine/cysteine
Pseudouridine (1)		
Allopurinol (3)	<i>3-Methylhistidine</i> (1)	
Guanine (2)		CHES
Uridine (1)		
Guanosine (1)		
d-Guanosine (1)	<i>α-Alanine</i> (2)	Glycine/leucine/valine
Adenine (2)		
Theobromine (1)		<i>β-Alanine/OH⁻</i> (terminator)

*Chemicals were purchased from Sigma (1), Merck (2) and Wellcome (London, Great Britain) (3).

**ACES = 2-(2-amino-2-oxyethylamino)ethanesulfonic acid; BES = N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid; CHES = 2-(N-cyclohexylamino)ethanesulfonic acid; EPPS = N-2-hydroxyethylpiperazinepropanesulfonic acid; HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES = 2-morpholinoethanesulfonic acid; MOPS = morpholinopropanesulfonic acid; TAPS = tris-(hydroxymethyl)-methylaminopropanesulfonic acid; TES = N-[tris-(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; TRICINE = N-[tris-(hydroxymethyl)-methyl]-glycine. All chemicals were of analytical grade.

3 µl. No further sample pretreatment was carried out. The non-UV-absorbing compounds that were found to be useful as spacers, are listed in italics in Table II.

Two different analyses of the same standard mixture are shown in Fig. 2. In Fig. 2A a separation without spacers is shown; Fig. 2C shows that with the

spacers the various UV-absorbing zones are more clearly visibly separated from each other. The UV trace of the blank run, i.e. the analysis of the spacer mixture, is shown in Fig. 2B. The standard mixture contained 17 UV-absorbing compounds and 8 spacers (Fig. 2C). Deoxyinosine, uridine and deoxyguanosine can also be separated; in the electrolyte system used (Table I) these compounds form adjacent zones behind inosine, guanine and guanosine, respectively. No appropriate spacers have been found until now. Discrimination of deoxyinosine and deoxyguanosine is possible with conductimetric detection. Unidentified

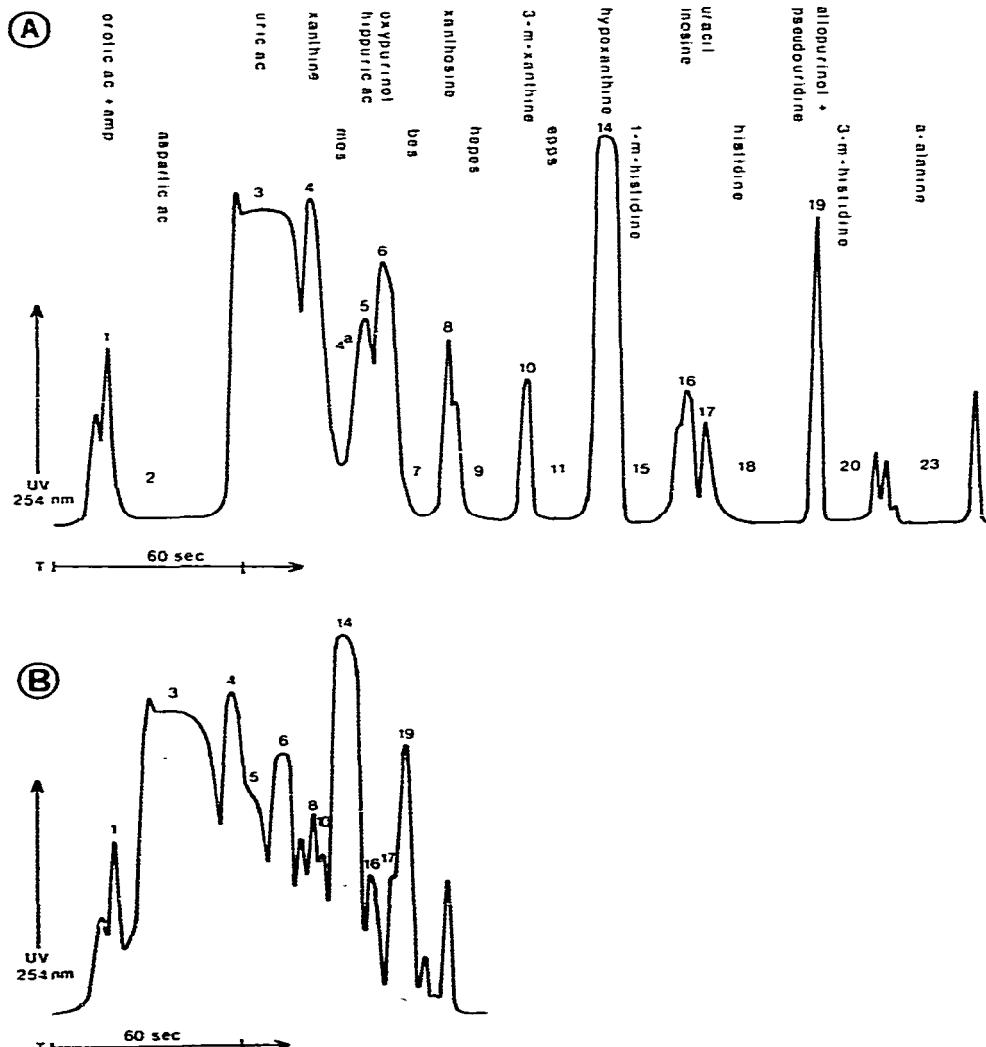


Fig. 3. UV traces of isotachophoretic analyses of urine from a Lesch-Nyhan patient under allopurinol treatment: (A) with spacers; (B) without spacers. Injected were 3 μ l of urine (diluted 1:5) and 1 μ l of spacer-mixture [a solution of aspartic acid (4 mM), MES (1 mM), BES (1.5 mM), HEPES (2 mM), EPPS (2 mM), 1-methylhistidine (2 mM), histidine (3 mM), 3-methylhistidine (2 mM) and α -alanine (2 mM)]. The numbered peaks are identified in Fig. 2.

substances normally occurring in urine can function as spacers [3]. In the case of uridine, the extinction ratio or the transmission ratio (for example, 280 nm and 254 nm) can also be used for identification. Pseudouridine forms a "steady-state" mixed zone with allopurinol in urine (Fig. 3). Adenosine and deoxyadenosine do not migrate in the electrolyte system (Table I) in the isotachophoresis stack (leading electrolyte-terminating electrolyte configuration) and consequently will not be detected. Severe deficiency of hypoxanthine-guanine phosphoribosyltransferase (HG-PRT, EC 2.4.2.8; see also Fig. 1) is mostly associated with a neurologic disease known as the Lesch-Nyhan syndrome [7]. One of the metabolic disturbances in this disease is increased purine

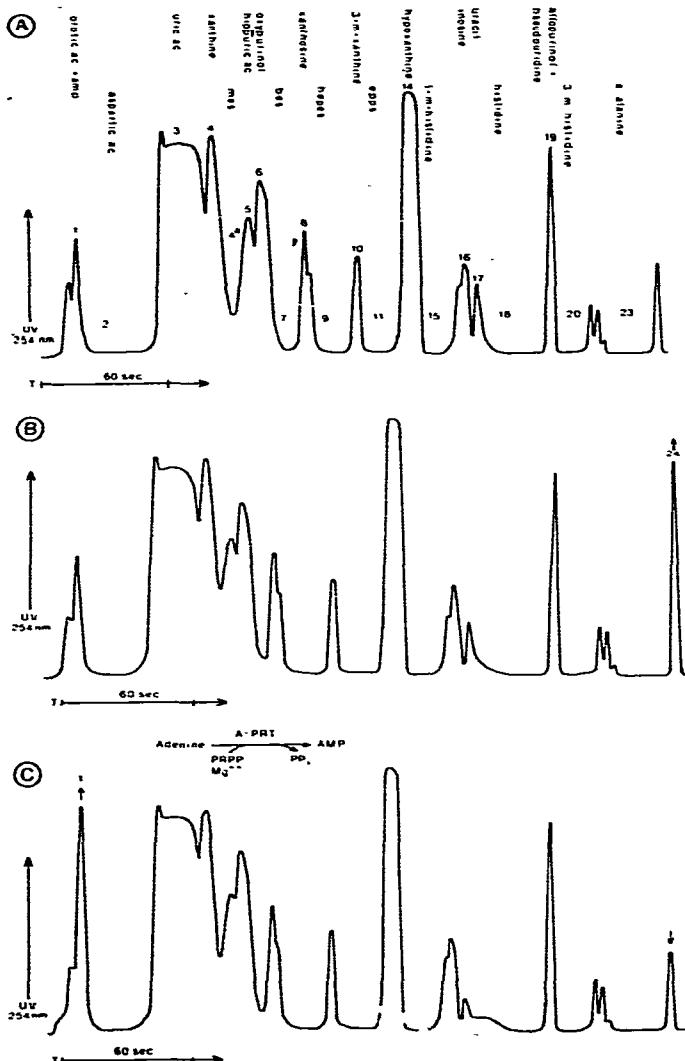


Fig. 4. Analysis of urine of a Lesch-Nyhan patient under allopurinol treatment: (A) unprocessed urine with spacers; (B) after adding 50 μ M adenine to the urine; (C) after preincubation of the same urine as in (B) with A-PRT. The numbered peaks are identified in Fig. 2. For the amount injected, see Fig. 3.

biosynthesis, resulting in hyperuricemia [8]. Consequently, urine from a Lesch-Nyhan patient contains a higher amount of uric acid as compared to urine from a normal control [3]. Fig. 3 shows UV scans of urine from a Lesch-Nyhan patient under allopurinol treatment. This drug reduces the amount of uric acid formed by inhibiting the xanthine oxidase reaction (see Fig. 1). This leads to the accumulation of the more soluble purine bases xanthine and hypoxanthine [3]. Allopurinol itself is converted to oxipurinol by xanthine oxidase, whereas small amounts of unchanged allopurinol are also excreted [3] (Fig. 3A and B). Addition of the various spacers allows a better discrimination between purines and pyrimidines (Fig. 3A). In Fig. 3B a run without any extra spacers is shown. In all further analyses the urine of the same allopurinol-treated Lesch-Nyhan patient was used.

Preincubation of a Lesch-Nyhan urine sample with purified A-PRT did not result in the disappearance of the zone at the place where adenine would be expected: a UV trace identical to that in Fig. 3A was obtained (Fig. 4A). Extra addition of adenine ($50 \mu M$) resulted in a clearly increased zone-length (Fig. 4B). After preincubation of this sample with A-PRT the adenine added was converted to AMP (Fig. 4C; see also Fig. 1). The original zone (Fig. 4A) was still present, indicating that this zone is definitely not adenine. PNP converts

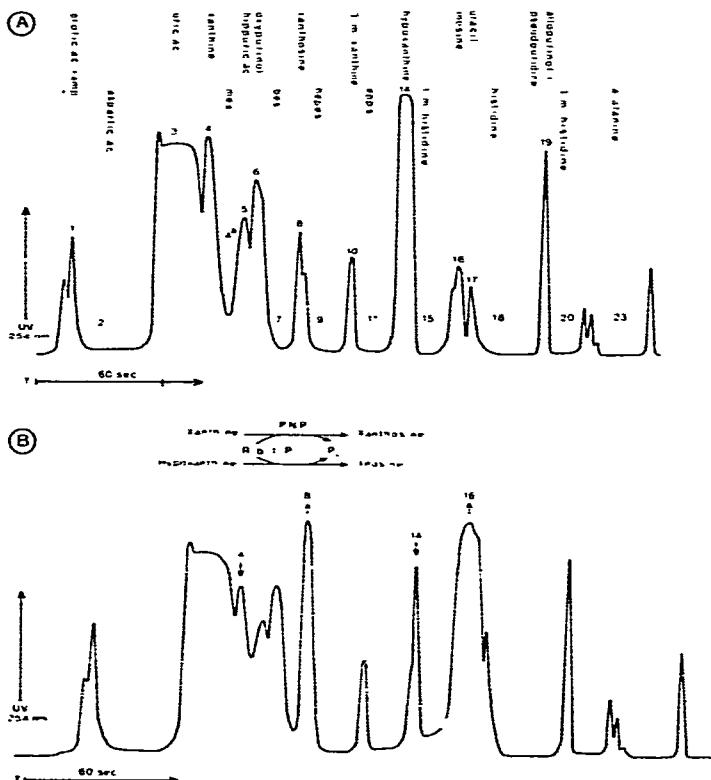


Fig. 5. Analysis of urine of a Lesch-Nyhan patient under allopurinol treatment: (A) unprocessed urine with spacers; (B) after preincubation of the urine with PNP. The numbered peaks are identified in Fig. 2. For the amount injected, see Fig. 3.

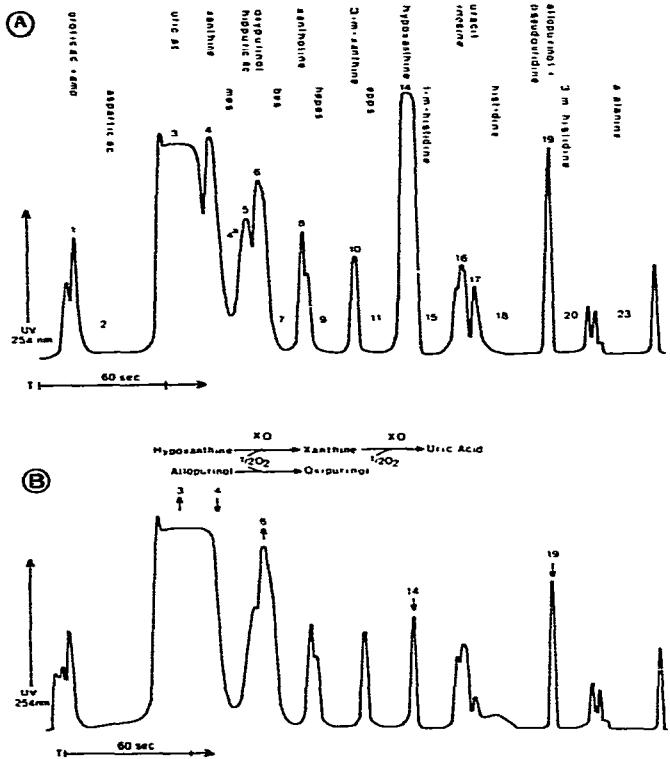


Fig. 6. Analysis of urine of a Lesch-Nyhan patient under allopurinol treatment: (A) unprocessed urine with spacers; (B) after preincubation of the urine with XO. The numbered peaks are identified in Fig. 2. For the amount injected, see Fig. 3.

hypoxanthine, xanthine and guanine in the presence of ribose-1-phosphate to inosine, xanthosine and guanosine, respectively (Fig. 1). The hypoxanthine zone decreased after preincubation with PNP, whereas the inosine zone increased*; the xanthine present was converted to xanthosine (Fig. 5A and B). No guanine was detected (Fig. 5A) and consequently no formation of guanosine was observed (Fig. 5B).

Xanthine oxidase acts on hypoxanthine and xanthine to form uric acid (Fig. 1). Both purine bases are present in the Lesch-Nyhan urine sample tested (Fig. 6A) and both disappeared after preincubation with xanthine oxidase (Fig. 6B). The uric acid zone increased (Fig. 6A and B). The free allopurinol which is present in the urine of the Lesch-Nyhan patient (Fig. 6A) is converted to oxipurinol by the action of xanthine oxidase (Fig. 6B).

The uricase reaction, which does not normally occur in human cells, removes the uric acid zone from the UV trace, giving rise to the formation of allantoin (Fig. 7B; see also Fig. 1), which runs ahead of hypoxanthine. It can be seen that after 22 h of preincubation uric acid is completely converted to allantoin (Fig. 7B).

*This zone is suspected of containing another UV-absorbing compound, which has not yet been identified.

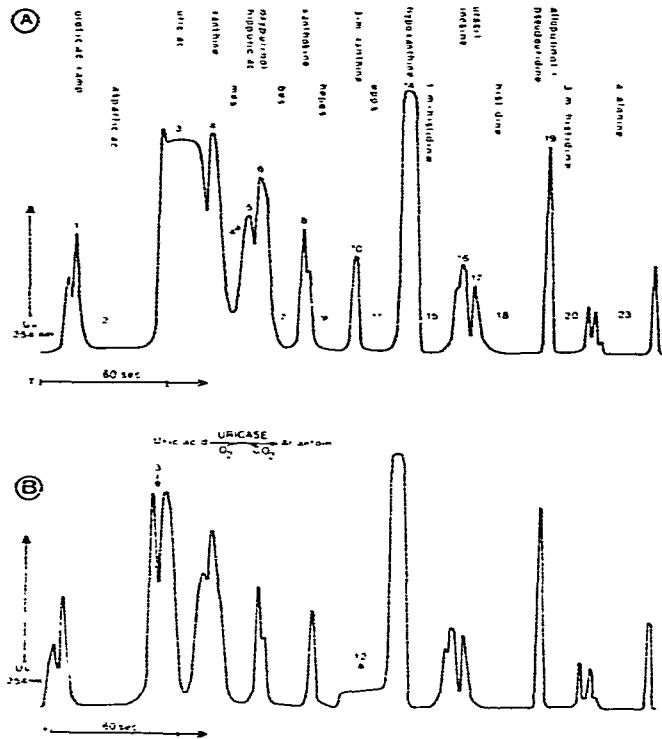


Fig. 7. Analysis of urine of a Lesch-Nyhan patient under allopurinol treatment: (A) unprocessed urine with spacers; (B) after preincubation of the urine with uricase. The numbered peaks are identified in Fig. 2. For the amount injected, see Fig. 3.

DISCUSSION

The isotachophoretic separation system presented in this paper offers a simple and rapid means of determining urinary purines and pyrimidines. The reproducibility is sufficiently high, variation coefficients being below 2%. In the present study, samples (ca. 3 μl) containing 17 nucleosides and bases of purines and pyrimidines could be separated conveniently within 20 min.

To obtain optimal results with the system given in Table I, several points should be considered: (1) the terminating electrolyte should be prepared freshly every day, filtered through a 0.22- μm Millipore filter and stored until use in closed electrolyte reservoirs (syringes) at room temperature; (2) after each run the terminator compartment must be emptied completely and refilled with fresh terminator; (3) the pH of the leading electrolyte should be checked every two runs and eventually be adjusted to pH 8.55 with ammediol. At a slightly deviating pH a poor separation of xanthine, hippurate and oxipurinol was obtained; (4) the counter-electrode compartment contains 5 mM HCl—ammediol (pH 8.55) (without hydroxyethylcellulose), and should be replenished every 4–5 runs; (5) the sample should be injected carefully into the leading electrolyte, due to the high pH of the terminating electrolyte.

This study concentrates on the analysis and identification of a number of purines and pyrimidines by means of spacers and enzymatic shifts. No attempts

were made to quantify the amounts of the various compounds. However, this can be done conveniently by measuring the integrated UV-absorbance peak area or zone length of a given compound [9].

The UV tracing of the electrolyte system, with spacers (Fig. 2B), showed several UV-absorbing and non-UV-absorbing zones, as could be anticipated. These compounds will also feature in the metabolite profiles and should accurately be discriminated from possible coincident sample zones. As evidenced by the findings shown in Fig. 3A and B, the use of non-UV-absorbing compounds as spacers (Table II) facilitates the interpretation of the metabolite profiles.

Possibilities of identifying a UV-absorbing compound include measurement of extinction ratio $\epsilon_{280}/\epsilon_{254}$ [3], and addition of the presumed compound ("spiking") and measuring the step height from the conductivity signal [2, 9, 10]. From Figs. 4-7 it follows that a specific and sensitive alternative is the enzymatic conversion of metabolites by purified enzymes.

The present isotachophoretic technique allows routine analyses of urinary purines and pyrimidines with a high degree of simplicity and reproducibility, for both diagnostic and experimental purposes.

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