ournal of Chromatography, 229 (1982) 67—75
iomedical Applications
lsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

HROMBIO. 1186

# IIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION )F PURINE AND PYRIMIDINE BASES, RIBONUCLEOSIDES, DEOXY-IBONUCLEOSIDES AND CYCLIC RIBONUCLEOTIDES IN BIOLOGICAL 'LUIDS

ONNEY A. DE ABREU* and JOHN M. VAN BAAL

*ivision of Pediatric Oncology, Department of Pediatrics, St. Radboud Hospital, Catholic Iniversity of Nijmegen, Nijmegen (The Netherlands)*

HRIS H.M.M. DE BRUYN

*epartment of Human Genetics, St. Radboud Hospital, Catholic University of Nijmegen, lijmegen (The Netherlands)*

nd

AN A.J.M. BAKKEREN and EGBERT D.A.M. SCHRETLEN

*ivision of Pediatric Oncology, Department of Pediatrics, St. Radboud Hospital, Catholic Iniversity of Nijmegen, Nijmegen (The Netherlands)*

First received October 19th, 1981; revised manuscript received November 24th, 1981)

UMMARY

A method is presented for the separation and quantitative determination of compounds ormally related to purine and pyrimidine metabolism in biological material. The retention ehaviour of nucleobases, ribonucleosides, deoxyribonucleosides and cyclic ribonucleotides as been systematically investigated by reversed-phase high-performance liquid chromatography using a non-linear gradient. Ultimately a separation of the purine and pyrimidine ompounds was achieved in a 35-min run with an average detection limit of 5—10 pmol er injection. Recoveries of standards added to urine, plasma or serum were 96 ± 5%.

## NTRODUCTION

A variety of studies has revealed disturbances in purine and pyrimidine netabolism in inborn errors of metabolism [1—4], immune diseases [5—10] nd leukemia [11—14]. Analysis of purine and pyrimidine metabolites may llow the pathological mechanisms leading to the clinically observed diseases

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to be elucidated. In addition, measurements of the pools of these metabolites may contribute to more refined diagnosis and further therapeutic approaches.

The present reversed-phase high-performance liquid chromatographic (HPLC) method was designed to determine the pools of nucleobases, nucleosides and cyclic nucleotides in urine, serum and plasma samples. Recently, several HPLC procedures have been presented with sensitive separations of nucleobases and nucleosides [15—19]. The HPLC procedure presented here offers the advantage of measuring various nucleobases, ribonucleosides, deoxyribonucleosides and cyclic nucleotides in a single run.

## EXPERIMENTAL

### *Chemicals*

All nucleobases, nucleosides, cyclic AMP and cyclic GMP used for the identification and quantitation of peaks in the UV scans were obtained from Sigma, St. Louis, MO, U.S.A., and from Boehringer, Mannheim, G.F.R. Helium was purchased from Hoekloos, Schiedam, The Netherlands. The other chemicals were from E. Merck, Darmstadt, G.F.R. All chemicals were of pro analysis grade. The water used for the buffer was purified in a Milli-Q System, giving a resistivity of 18 MΩ/cm (Millipore, Bedford, MA, U.S.A.).

### *Apparatus*

The experiments were performed with a Spectra-Physics SP8000 liquid chromatograph (Spectra Physics, Santa Clara, CA, U.S.A.). The apparatus was equipped with a thermostated oven, an auto-injector with a high-pressure sampling Valco valve, an automated data system with integrator, and a two-channel printer-plotter.

Column effluents were monitored with two UV detectors (SP8210, Spectra Physics), one set at wavelength 254 nm and the other at wavelength 280 nm. The flow-cells had volumes of 8 μl.

Pre-packed analytical columns 250 mm × 4.6 mm I.D. were used, packed with Spherisorb 10-ODS (particle size 10 μm; Chrompack, Middelburg, The Netherlands).

### *Chromatographic conditions*

Elutions were performed with a programmed non-linear (NL 2) ternary gradient, starting with 0.05 mol/l potassium phosphate buffer (pH 5.60) and ending with 50% 0.05 mol/l potassium phosphate buffer (pH 5.60), 25% methanol and 25% water (v/v). Before use the phosphate buffer was filtered through a Millipore Type HA membrane filter (pore size 0.45 μm). All mobile phases were degassed by continuous helium purging. The solvent flow-rate was kept constant at 1.5 ml/min, the pressure at ± 38 bars and the temperature at 40°C. The run-time was 35 min.

### *Sample preparation*

Urine samples were filtered through a Millipore Type GS membrane filter (pore size 0.22 μm) and were analysed immediately afterwards.

Serum and plasma samples were deproteinated with perchloric acid (PCA)

at 0°C. Serum or plasma (1 ml) was pipetted into a micro test-tube (Model 3810, Eppendorf, Hamburg, G.F.R.) and kept in an ice-bath. Then 100 $\mu$l of 4 mol/l ice-cold PCA was added and the mixture was vortexed for 1 min. The suspension was kept on ice for another 5 min and centrifuged in an Eppendorf centrifuge (Model 3200). After centrifugation, the supernatant was pipetted into another micro test-tube and the pH was adjusted to 6.0—6.8 with an ice-cold mixture of 4 mol/l potassium hydroxide and 1 mol/l dipotassium hydrogen phosphate. After 10 min the precipitated potassium perchlorate was removed by centrifugation and the extract was kept on ice until injection. If the extract was not investigated the same day, it was kept frozen at −23°C.

## RESULTS AND DISCUSSION

### *Chromatography*

Fig. 1 illustrates a typical analysis of a standard solution containing several purine and pyrimidine bases, ribonucleosides, deoxyribonucleosides, cyclic

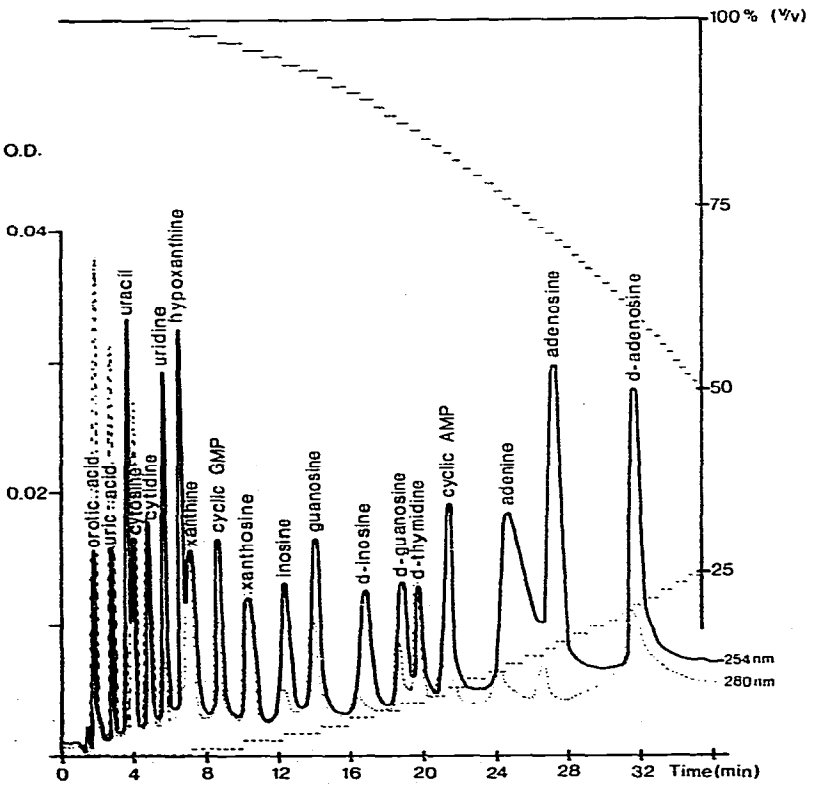



**Fig. 1. Elution profile of a test mixture of purine and pyrimidine nucleobases, ribonucleosides, deoxyribonucleosides, cyclic AMP and cyclic GMP. The separation conditions are described in Experimental. Detection was performed at 254 nm and 280 nm. On the scale to the right the percentage of each of the solvents used for the elution is indicated. The 0.05 mol/l potassium phosphate buffer starts at 100% and ends at 50% (indicated by the step-plot with solid lines), whereas the water and methanol each start at 0%, and end at 25% (indicated by the step-plot with broken lines).**

AMP and cyclic GMP. The best separation on the Spherisorb 10-ODS columns was achieved using the preprogrammed non-linear gradient described under Experimental. Between two successive runs the system was equilibrated for 15 min under the initial conditions. Some overlap was observed between cytosine and uracil and between hypoxanthine and xanthine. In these cases the absorbance ratios ($E_{254nm}/E_{280nm}$) of the partially overlapping compounds vary significantly enough to distinguish between the compounds.

*Recovery*

Standard amounts of purines and pyrimidines were added to human serum (50, 100, 200, 400, 800 and 1000 pmol/ml). At least five samples at each concentration were deproteinated as described under Experimental and analysed. The concentrations and the integrated peak areas were linearly related over the range 50—1000 pmol/ml. For cytosine, uracil, hypoxanthine and xanthine, correlation coefficients were found of 0.987, 0.990, 0.992 and 0.986, respectively. The correlation coefficients found for the other compounds were at least 0.996. The recoveries of all the standards added to serum were 96 ± 5%.

*Applications*

An application of the present method to biological material is illustrated in Fig. 2A and B. In this figure the UV scans of urine samples are shown from a patient with Lesch-Nyhan syndrome before (Fig. 2A) and after (Fig. 2B) receiving the drug 4-hydroxypyrazolo[3,4-*d*]pyrimidine (allopurinol). The Lesch-Nyhan syndrome is associated with severe deficiency of the enzyme hypoxanthine—guanine phosphoribosyltransferase (HGPRT; EC 2.4.2.8) [2, 20, 21]. The HGPRT deficiency leads to a massive over-production and over-excretion of uric acid [2, 21]. The drug allopurinol is an analogue of hypoxanthine. It is a competitive inhibitor in the formation of the poorly soluble uric acid from hypoxanthine and xanthine, a reaction catalysed by xanthine oxidase. Allopurinol is converted to oxipurinol by xanthine oxidase. As a result, allopurinol is an important agent in the control of hyperuricemia, by giving rise to the formation of the more soluble hypoxanthine and xanthine.

With the HPLC method the effect of allopurinol treatment on uric acid, hypoxanthine and xanthine excretion can easily be studied. In addition, the excreted amounts of free allopurinol and of produced oxipurinol can be detected, so the therapy can be adapted (Fig. 2A and B). Also eventual side-effects on pyrimidine metabolism can be monitored.

In other investigations we are using the HPLC method in the diagnosis of patients with pseudohypoparathyroidism. This is an hereditary disorder, characterised by symptoms and signs of hypoparathyroidism in association with distinctive skeletal and developmental defects [22—26]. The cause of this disease differs, however, from that of hypoparathyroidism. In the latter cases there is deficient parathyroid hormone (PTH) production. In pseudohypoparathyroidism there is excessive secretion of PTH. However, there is a defective response of target-cell receptors or of adenyl cyclase to PTH. Therefore, two principal laboratory tests should be employed, namely the measurement of PTH secretion and the measurement of urinary cyclic AMP excretion following the administration of PTH. In Fig. 3A and B, UV scan patterns are

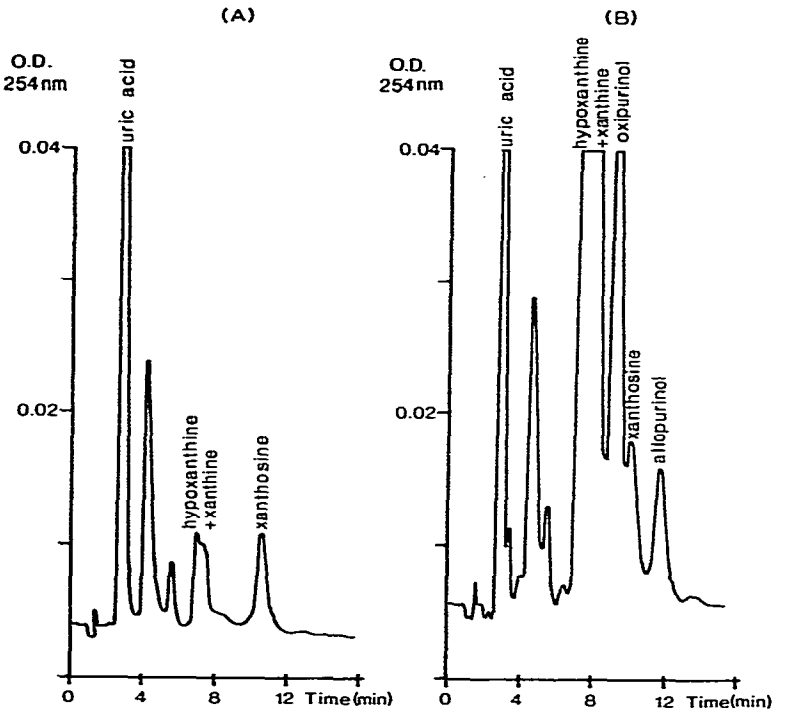



**Fig. 2. UV scans of urine samples from a Lesch-Nyhan patient before (A) and after 5 days (B) of treatment with an oral dose of allopurinol, 10 mg/kg body weight per 24 h.**

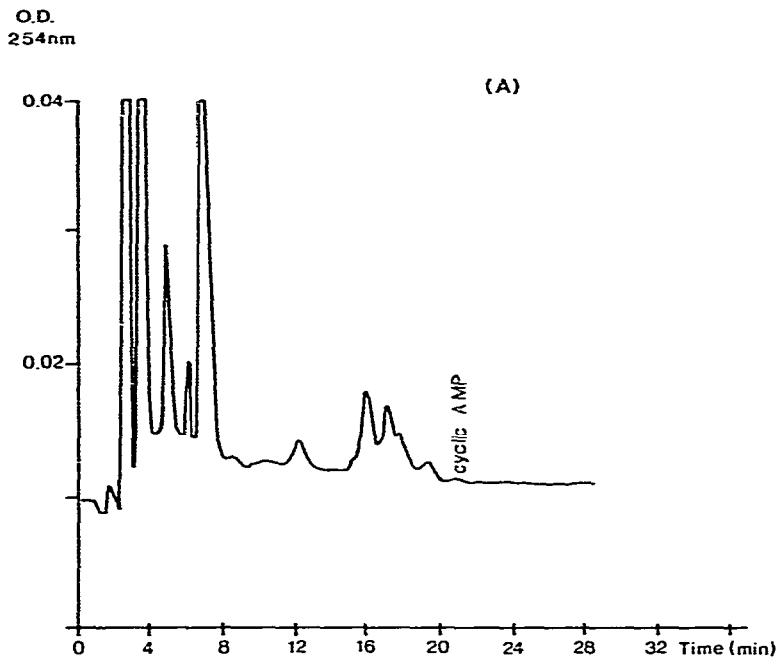
shown of urine from a normal healthy control before and 30 min after administration of PTH. In Fig. 3C and D, UV scan patterns are illustrated from a patient suspected of having pseudohypoparathyroidism before and 30 min after administration of PTH. In contrast to the control, no increase in the cyclic AMP levels was observed in the patient, suggesting that this patient had the disease.

The HPLC system is also used in in-vitro studies of nucleobase and nucleoside metabolism of malignant human T- and B-cell cultures. In this way a more detailed understanding can be obtained of the differences in purine and pyrimidine metabolism known to occur in lymphoblasts of various subsets of acute lymphatic leukemia [11—14]. In Fig. 4A and B an example of such an experiment is given with the Raji cell-line (human Burkitt's lymphoma B-cell). The culture medium was analysed before and after addition of 100 μmol/l adenosine. The major metabolite after transport and metabolism appears to be hypoxanthine (Fig. 4B).

## CONCLUSIONS

The HPLC method reported is a useful and accurate analytical tool to determine concentrations of purine and pyrimidine metabolites in a variety of body fluids.

In comparison to previously published methods [15—19], the advantage of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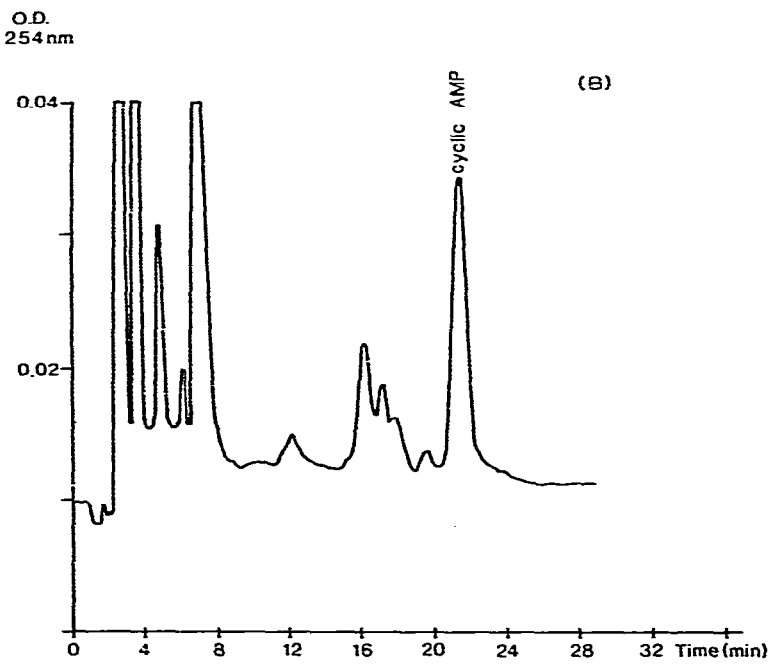
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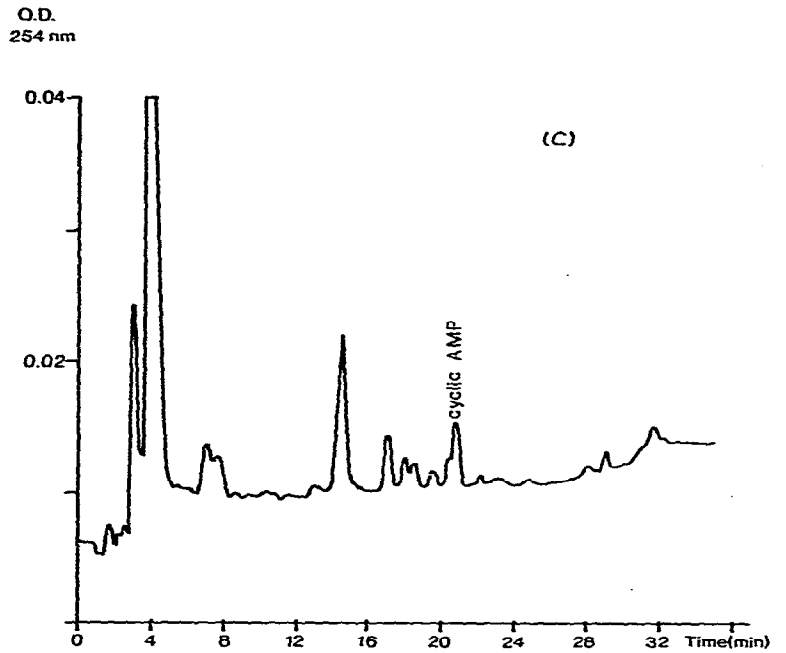
Fig. 3.



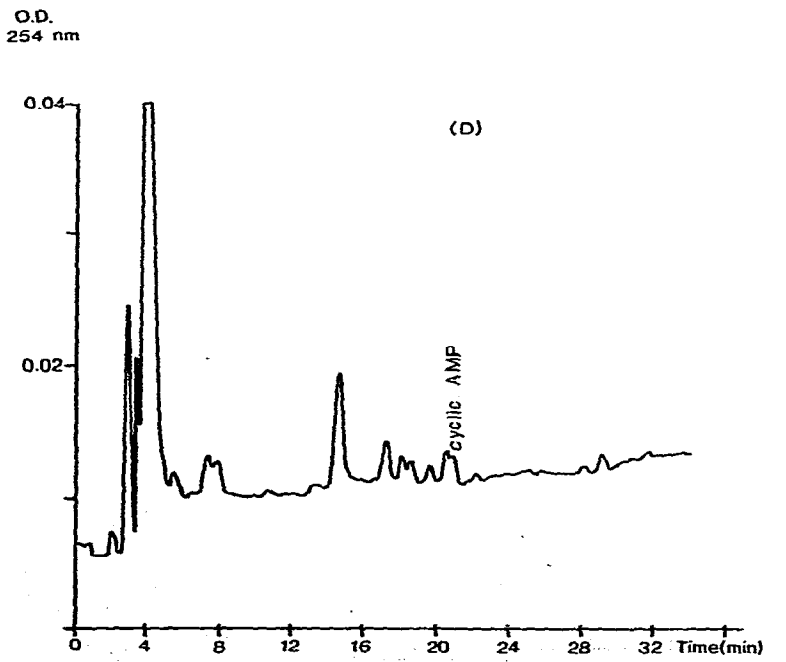




**Fig. 3. Excretion of 3',5'-cyclic AMP following an injection of PTH (300 U S.P. units). (A) Urine of a healthy control before administration of PTH. (B) Urine of the control 30 min after the administration. (C) Urine of a patient with pseudohypoparathyroidism before administration of PTH. (D) Urine of the patient 30 min after administration of PTH.**

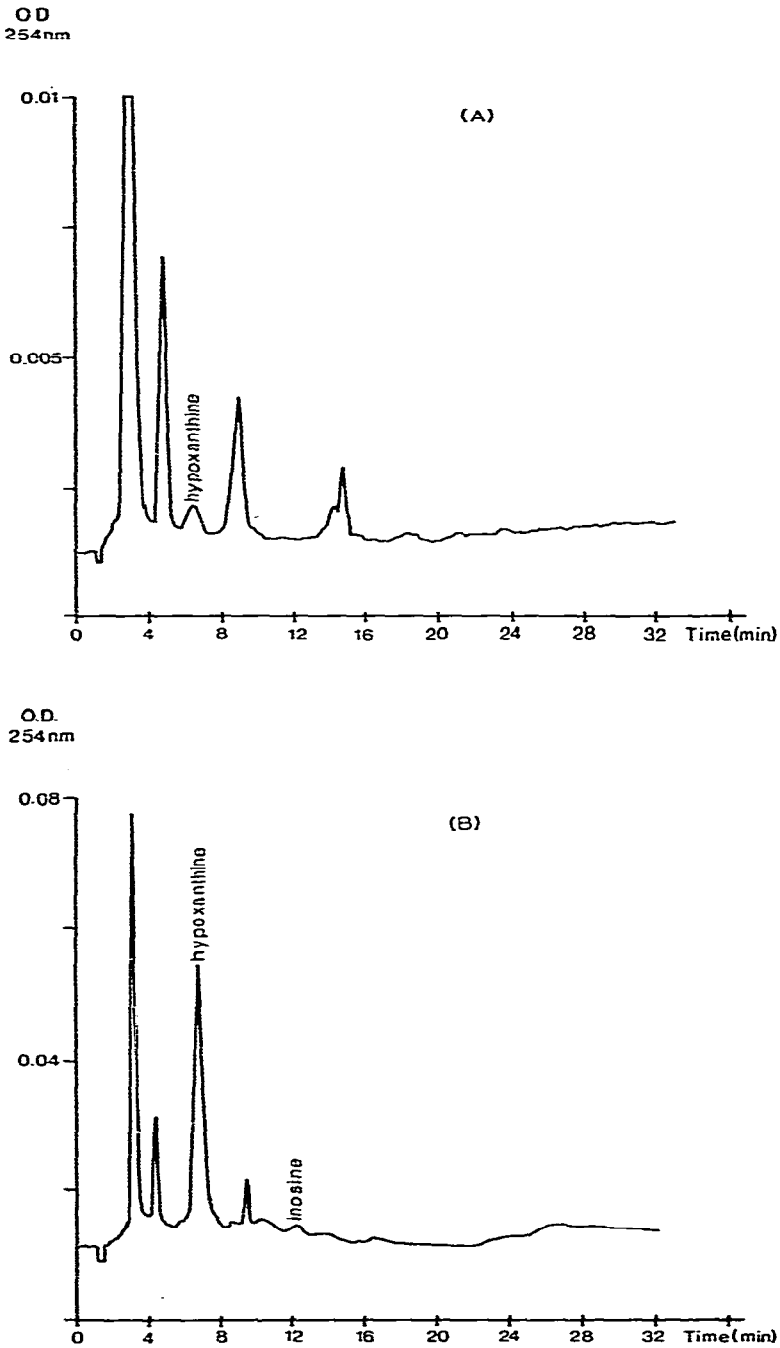



**Fig. 4. Elution profile of purine and pyrimidine nucleosides in a culture medium before (A) and 15 h after (B) addition of 100 μmol/l adenosine to a cell culture of a Raji cell-line.**

the method presented is that it enables the concentrations of nucleobases, ribonucleosides, deoxyribonucleosides, cyclic AMP and cyclic GMP to be determined in a single run.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Queen Wilhelmina Fund for Cancer Research in The Netherlands. The authors are grateful to Dr. J. van Laarhoven and M. Tilanus (Department of Human Genetics) for valuable discussions.

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