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ON-LINE HIGH-PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY–HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF MONOMERIC RIBONUCLEOSIDE COMPOUNDS IN BIOLOGICAL FLUIDS

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SUMMARY

We describe an on-line multi-dimensional column chromatography system. It consists of a high-performance liquid affinity chromatography prefractionation column for *cis*-diol compounds and a series-connected reversed-phase high-performance liquid chromatography system for the analysis of ribonucleosides. This on-line procedure allows the rapid and direct analysis of methylated ribonucleic acid catabolites in biological fluids (serum, urine) and therefore might be useful in pathobiochemistry.

INTRODUCTION

Borate is known to form complexes with polyols under alkaline conditions[1,2]. Linked to polymer matrices, *e.g.* agarose, cellulose, or polyacrylamide, boronic acid has been used as ligand in affinity chromatography of *cis*-diol-containing compounds such as carbohydrates, catecholamines, ribonucleosides, ribonucleotides and ribonucleic acids (mRNA, rRNA, tRNA)[3–7]. Metabolic degradation of these RNA yields modified monomers which should represent sensitive indicators of RNA metabolism. Thus, the measurement of altered patterns of modified RNA catabolites in serum and urine may be useful for the diagnosis and treatment of pathobiochemical processes. Moreover, comparison of the serum and urinary nucleoside patterns should allow a convenient kidney-function test of the elimination and reabsorption of nucleosides.

Up to now, the detection of such biological markers in urine and serum required time-consuming sample preparation and prefractionation steps[4–6]. For routine analysis of biological fluids, however, a rapid and direct on-line multi-dimensional column chromatography system would be the ultimate goal.

As liquid affinity chromatography (LAC) has been demonstrated to work under high pressure, introducing a method named high-performance liquid affinity chromatography (HPLAC)[8], we have tried to use this procedure for the prefractionation step. We have exploited the HPLAC system by covalent fixation of the *cis*-diol-specific ligand *m*-aminophenylboronic acid to silica (Scheme I)[9]. By coupling the

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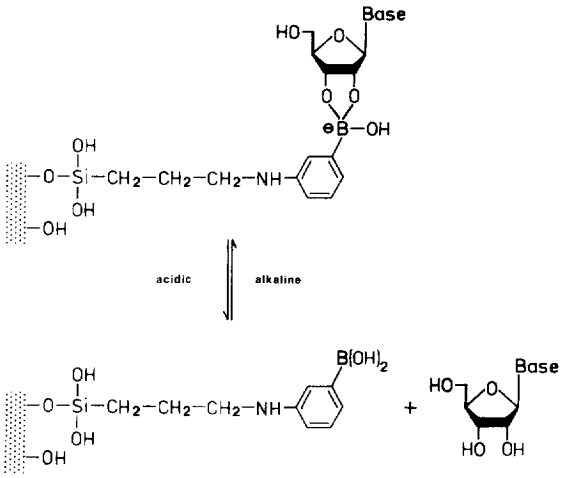


Scheme I.

HPLAC system to a reversed-phase high-performance liquid chromatography (RP-HPLC) system we finally came up with a powerful and sensitive on-line chromatography system for the analysis of *cis*-diols in biological fluids. Because applications of boronate in affinity chromatography are numerous, further exploitation of our on-line system for different fields of research appears to be very promising.

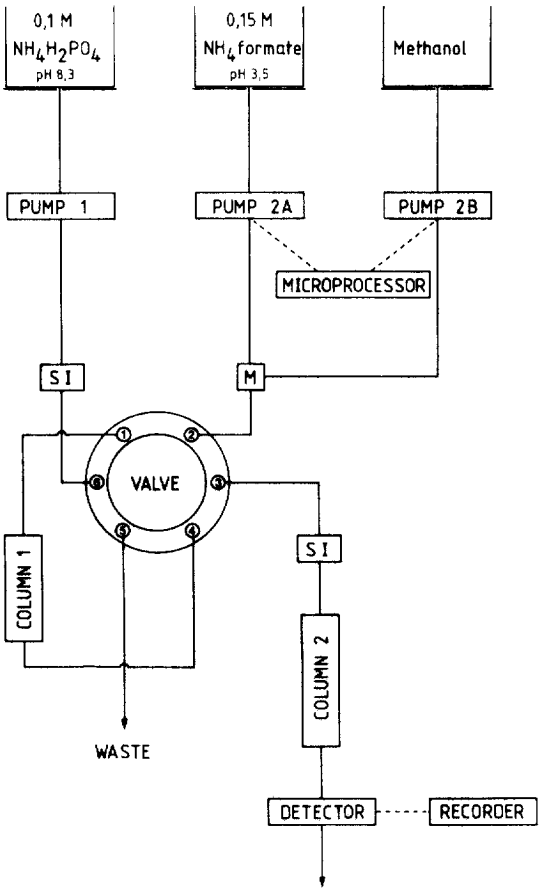
EXPERIMENTAL

Apparatus

A basic Altex microprocessor-controlled gradient system (Altex, Berkeley, CA, U.S.A.), consisting of two Altex Model 110A pumps, a Model 420 microprocessor, a Rheodyne Model 7125 loop injector for sample introduction and a Kontron Uvicon Model 725 spectrophotometer, was additionally equipped with a third Altex Model 110A pump, a second Rheodyne 7125 loop injector and a Rheodyne Model 7010 six-port valve, as shown in Scheme II. Integration was performed with a Hewlett-Packard Model 3390A integrator (Hewlett-Packard, Avondale Division, PA, U.S.A.).

Analytical procedure

For the direct on-line analysis of ribonucleosides in urine and serum HPLAC Column 1, filled with boronic acid-substituted silica, was equilibrated for 2 min in valve position "Load" with 0.1 *M* ammonium phosphate (pH 8.3). After sample injection (20–100 μl of urine, deproteinized serum, or a synthetic mixture), Column 1 was washed for 2 min with the same buffer. During that time ribonucleosides were selectively retarded on the HPLAC column and the sample matrix was discharded (Scheme III). After this clean-up step the valve was switched to "Inject" and thereby series-connected in front of Column 2 (Scheme III). The group-specifically bound ribonucleosides on Column 1 were then eluted under acidic conditions (0.15 *M* ammonium formate, pH 3.5) in a small volume (*ca.* 700 μl) through position 2-1-4-3 of



Scheme II.

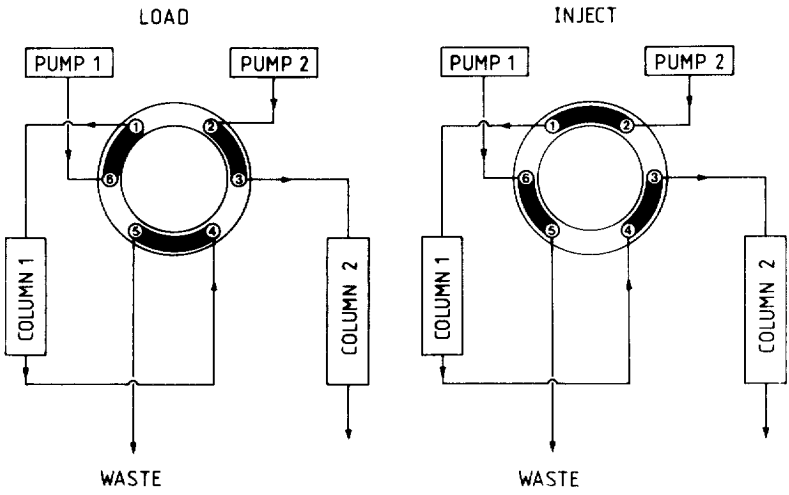
the valve (Scheme III) and concentrated on top of the reversed-phase C_{18} column over a period of 1.5 min. This elution buffer was also used for the subsequent analytical separation. The valve, was then switched back into position "Load" (Scheme III) and elution of Column 2 could be carried out independently in the traditional way by increasing the amount of organic modifier in the mobile phase (chromatographic conditions, *cf.* Fig. 1).

Columns

The HPLAC column (Column 1; 30 × 4 mm I.D.) was filled by an upward slurry-packing technique with a laboratory-prepared boronic acid-substituted silica[9]. Column 2 (250 × 5 mm I.D.) was laboratory-packed with LiChrosorb RP-18, 7 μm (Merck, Darmstadt, F.R.G.).

Sample preparation

For routine analysis 1 ml of human urine was membrane-filtered (Millox 0.22 μm; Millipore, Buc, France) and an aliquot of 20 μl was applied to the HPLAC column. Samples (100 μl) of human serum were adjusted to pH 4 with formic acid



Scheme III.

and deproteinized within 10 min by centrifugal ultrafiltration (Amicon, Micropartition System, Witten, F.R.G.). A 20-μl volume of the ultrafiltrate was applied to the HPLAC column.

Materials

The ribonucleosides adenosine (Ado), cytidine (Cyd), inosine (Ino), uridine (Urd) and guanosine (Guo) were purchased from Boehringer (Mannheim, F.R.G.). The methylated ribonucleosides N^1-methyladenosine (m^1Ado), N^1-methylinosine (m^1Ino), N^1-methylguanosine (m^2Guo), N^6-dimethyladenosine (m^6_2Ado), pseudouridine (ψ) were from Sigma (München, F.R.G.). N^2-dimethylguanosine (m^2_2Guo) and N^6-(carbamoyl-threonyl)-adenosine (t^6Ado) were isolated from urine. For further characterisation, t^6Ado was also chemically synthesized[10].

RESULTS AND DISCUSSION

The conventional clean-up procedure for ribonucleosides in urine and serum, including step-wise pH gradient elution and group-specific prefractionation under low-pressure liquid chromatography (LAC) conditions can now be performed under high pressure conditions thanks to the development[9] of a suitable HPLAC column. Under acidic conditions, the ribonucleosides investigated are eluted from the HPLAC column in a small volume (*ca.* 700 μl). This allows a column-switching technique, *i.e.* the direct transfer of this zone to a reversed-phase column. In addition, most nucleosides are concentrated on top of the RP-C_{18} column due to their hydrophobic character. After this transfer step RP-HPLC can be performed independently, and elution of ribonucleosides can be carried out by increasing the amount of organic modifier in the mobile phase (Fig. 1).

A comparison of the RP-HPLC method (Fig. 1a) with the on-line procedure (Fig. 1b–d) shows that the latter does not affect band-broadening and resolution of the nucleosides investigated. This result is in good agreement with the findings of Gehrke *et al.*[11], who demonstrated that sample volumes up to 1 ml do not essentially

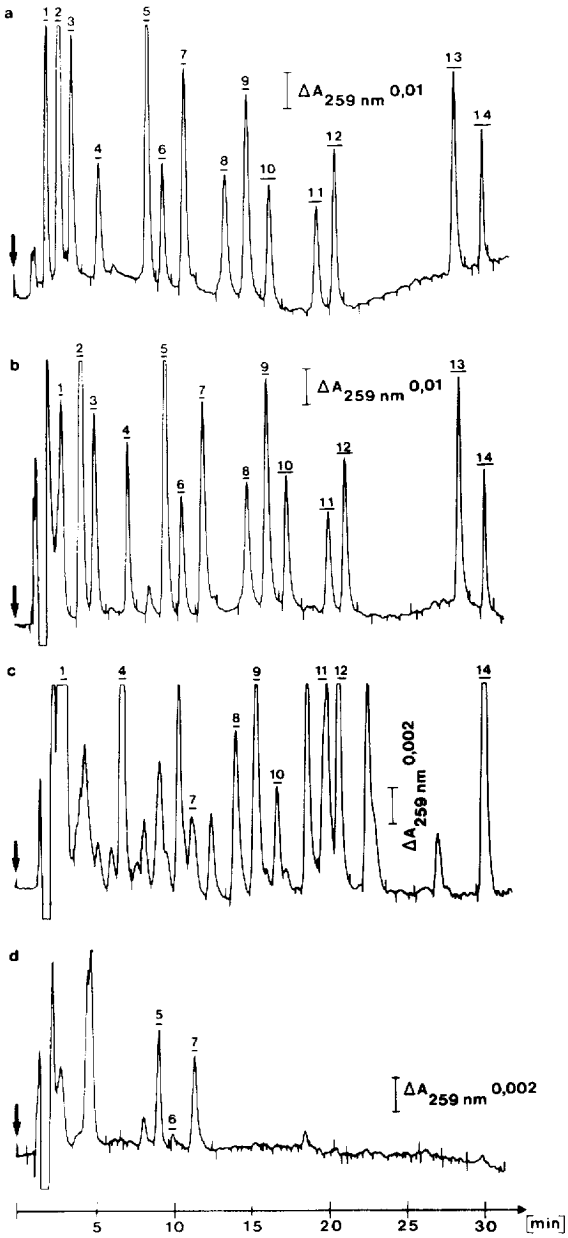


Fig. 1. (a) RP-HPLC of a synthetic mixture of fourteen ribonucleosides. 1 = Pseudouridine (ψ); 2 = uridine (Urd); 3 = cytidine (Cyd); 4 = N^1-methyladenosine (m^1Ado); 5 = inosine (Ino); 6 = guanosine (Guo); 7 = adenosine (Ado); 8 = N^1-methylinosine (m^1Ino); 9 = N^1-methylguanosine (m^1Guo); 10 = N^2-methylguanosine (m^2Guo); 11 = N^6-methyladenosine (m^6Ado); 12 = N^2-dimethylguanosine (m^2_2Guo); 13 = N^6-dimethyladenosine (m^6_2Ado); 14 = N^6-(carbamoyl-threonyl)-adenosine (t^6Ado). (b) On-line analysis of the same synthetic mixture of nucleosides. (c) and (d) On-line analysis of 20 μl each of membrane-filtered urine and serum ultrafiltrate, respectively. Chromatographic conditions: elution of column 2, 0.15 *M* ammonium formate (pH 3.5); after 2 min, linear gradient up to 8% methanol in 10 min followed by linear gradient up to 30% methanol in 20 min; flow-rate, 1.8 ml/min; detection, UV 259 nm. In (b)–(d) the HPLAC column (Column 1) was series-connected to the RP column during the first 1.5 min of elution (compare *Analytical procedure*).

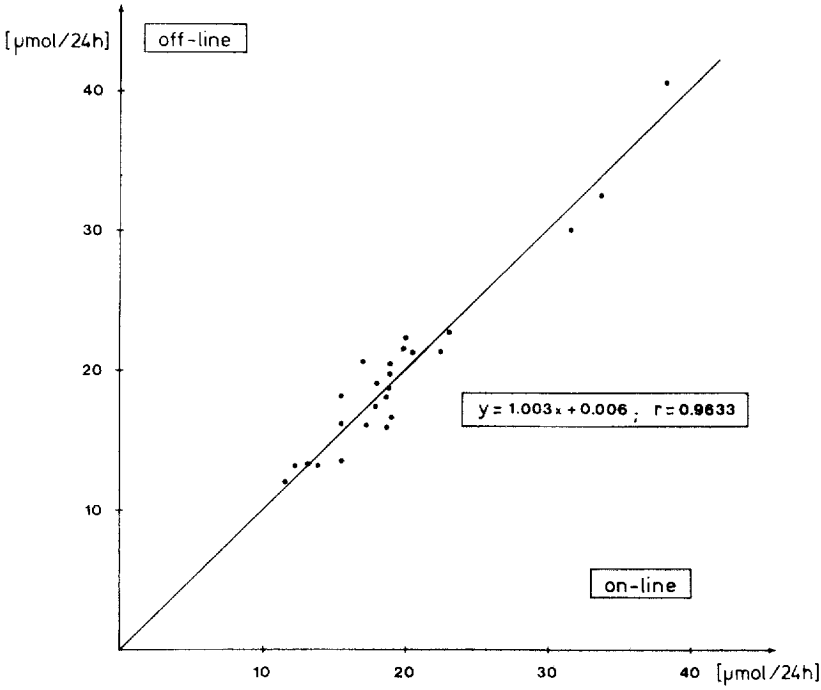


Fig. 2. Comparison of the off-line LAC–RP-HPLC and on-line HPLAC–RP-HPLC methods for the determination of m^1Ado in 25 different urines (each value mean of three independent analyses).

influence theoretical plate height and resolution of nucleosides on a RP-C_{18} column. Thus, the analytical separation of nucleosides primarily depends on the performance of the RP-column.

The method described allows the direct analysis of ribonucleosides in native urine or deproteinized serum within 40 min with a detection limit of 1 pmol of nucleosides per µl of biological fluid. The use of a volatile buffer and methanol as organic modifier facilitates the post-chromatographic structural analysis necessary for characterization of still unknown *cis*-diols in biological fluids.

The on-line HPLAC–RP-HPLC method described in this paper is compared with the conventional off-line LAC–RP-HPLC procedure[4] applied by us for the quantitative determination of m_2^2Guo, m^1Ino, m^1Ado and m^2Guo in urine of normal and pregnant females[1,2]. The results obtained by both methods show excellent agreement and good correlation (data shown in Fig. 2 for m^1Ado). The on-line system is the method of choice, as (a) it greatly decreases the analysis time, (b) it is suitable for automation and (c) in conjunction with a data-processing system, it is applicable to routine clinical analysis.

NOTE ADDED IN PROOF

Recently, Serva Feinbiochemica (Heidelberg, F.R.G.) developed a Dihydroboryl-Si 100 Polyol of comparable HPLAC quality.

ACKNOWLEDGEMENT

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