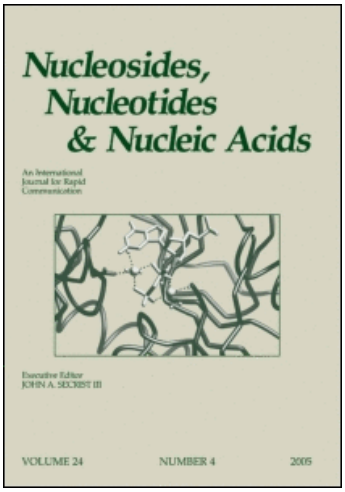

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An Automated Coupled-Column HPLC-System for the Direct Analysis of Ribonucleosides and Ribonucleotides in Biological Fluids

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AN AUTOMATED COUPLED-COLUMN HPLC-SYSTEM FOR THE DIRECT ANALYSIS OF RIBONUCLEOSIDES AND RIBONUCLEOTIDES IN BIOLOGICAL FLUIDS

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Abstract: We developed a coupled dual column HPLC-system for on-line sample processing, trace enrichment and analysis of ribonucleosides and ribonucleotides. The fully automated HPLC-analyzer tolerates the direct and repetitive injection of biological fluids such as serum or urine by use of an unique bonded-phase precolumn material which allows the simultaneous performance of covalent affinity and size-exclusion chromatography.

Despite its high resolution power, sensitivity, precision and practicability, routine HPLC-analysis of ribonucleosides and ribonucleotides in biological samples like urine, serum, milk and supernatants of tissue homogenates has been restricted by the manual pretreatment and processing of such highly complex matrices.

By preparing an unique bonded-phase material <1>, we succeeded in the development of a commercially available HPLC-analyzer that quantifies ribonucleosides or ribonucleotides after the direct injection of an appropriate biological fluid <2,3>. The precolumn material is a chemically modified copolymer and allows the simultaneous performance of two different modes of liquid chromatography under high pressure conditions. First, by virtue of its size-

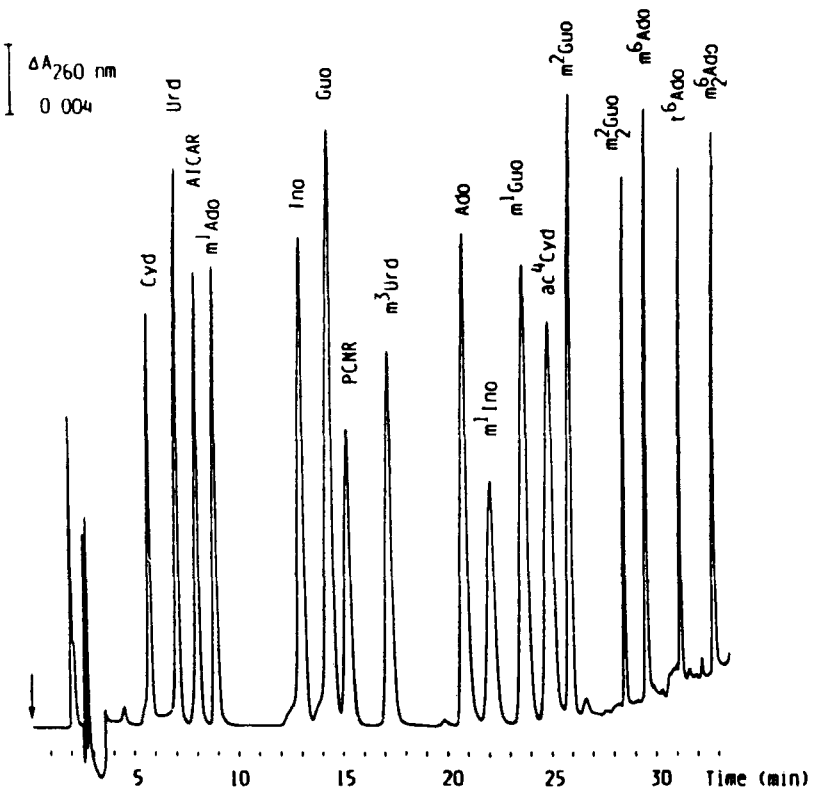

FIG. 1: Coupled-column analysis of a synthetic mixture of ribonucleosides. Sample volume: 100 µl; Compounds (nmol): AICAR (0.35); m^1Ado (0.81); Ino (2.06); Guo (1.83); PCNR (1.49); Ado (1.30); m^1Ino (1.88); m^1Guo (2.11); ac^4Cyd (1.05); m^2Guo (1.03); m^2_2Guo (0.47); m^6Ado (0.54); t^6Ado (0.53) m^6_2Ado (0.62). Taken from Ref. 2 with permission.

exclusion properties, macromolecules (e.g. proteins) and interfering non-analytes quantitatively can be separated from the solute. Secondly, by immobilizing a specifically modified phenylboronic acid to the gel support, covalent affinity chromatography can be performed. The chemoselective retention as well as trace enrichment is based on the pH-controlled formation or hydrolysis of cyclic boronate esters with the cis-diol groups of the analytes. The sample processing precolumn, which is connected with or separated from the analytical

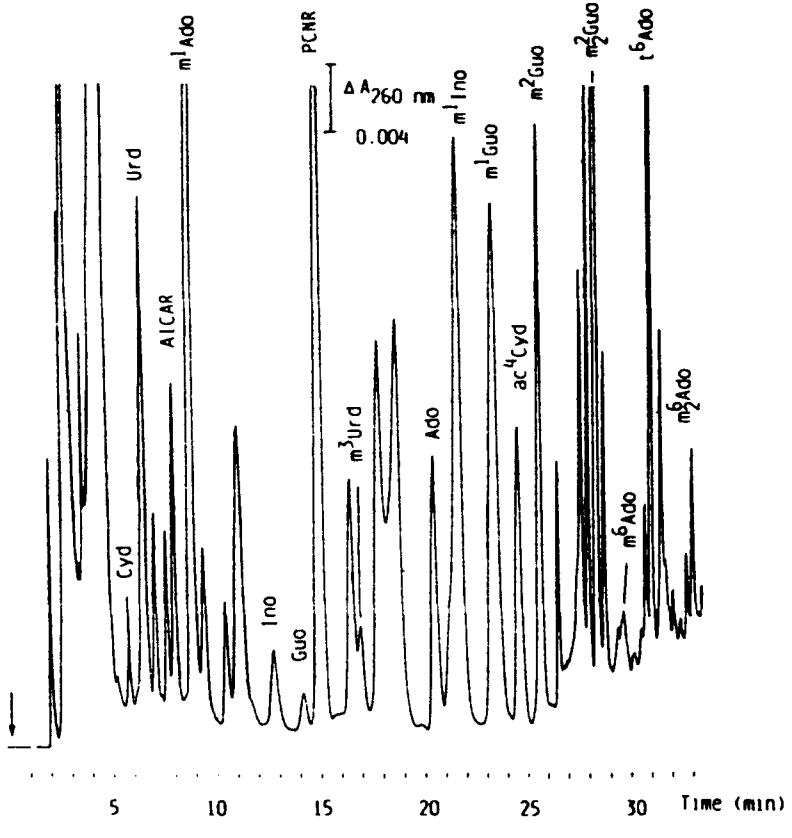

FIG. 2: Coupled-column analysis of ribonucleosides in normal human urine.
Sample volume: 100 µl; Compounds (nmol): AICAR (0.27); m^1Adc (4.17); Ino (0.42); Guo (0.09); PCNR (4.87); Ado (0.71); m^1Ino (5.43); m^1Guo (2.88); ac^4Cyd (0.59); m^2Guo (1.10); m^2_2Guo (4.21); t^6Ado (1.98) m^6_2Ado (0.09). Taken from Ref. 2 with permission.

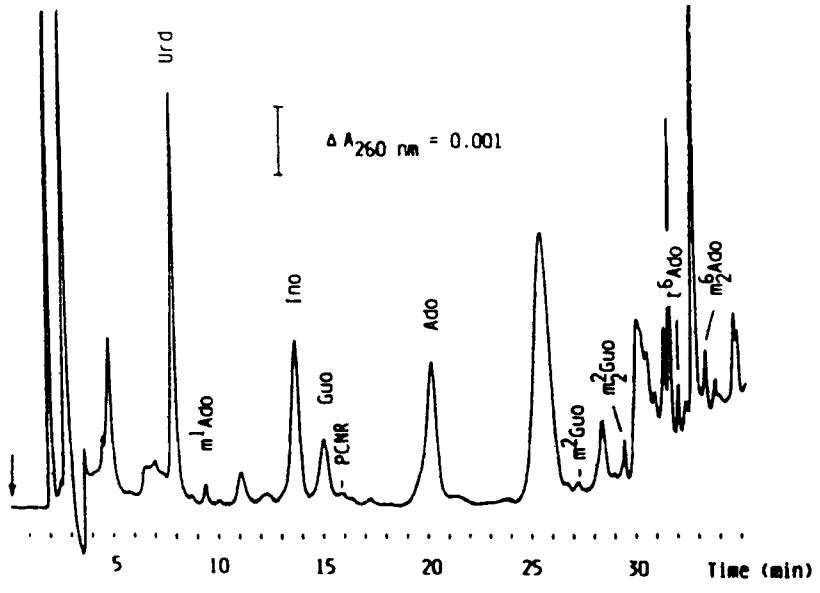

FIG. 3: Coupled-column analysis of ribonucleosides in 500 µl normal human serum. Compounds (pmol): m^1Ado (6); Ino (199); Guo (72); PCNR (17); Ado (174); m^2Guo (7); m^2_2Guo (14); t^6Ado (57); m^6_2Ado (31). Taken from Ref. 2 with permission.

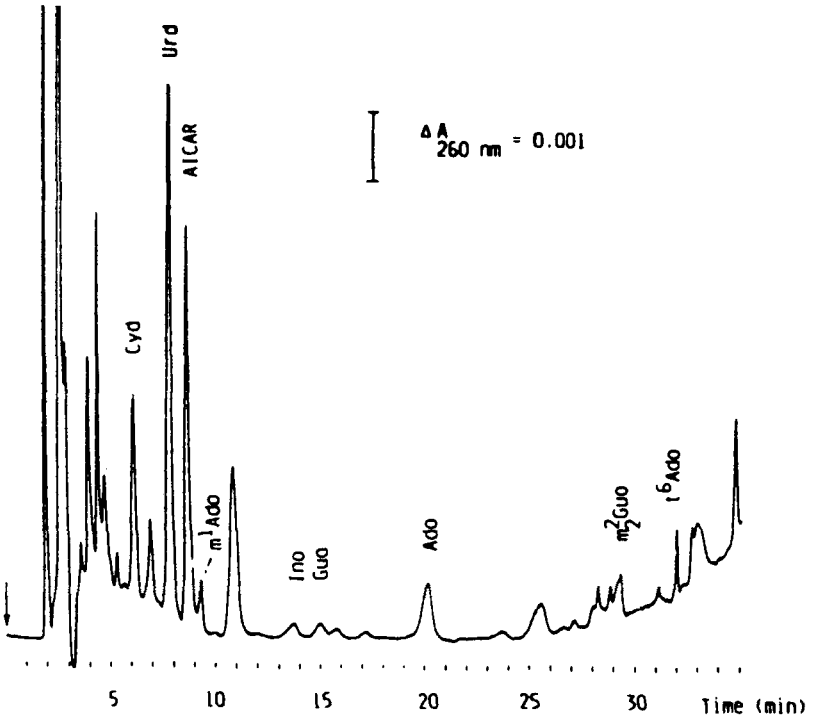

FIG. 4: Coupled-column analysis of ribonucleosides in 100 µl breast milk. Compounds (pmol): AICAR (84); m^1Ado (32); Ino (35); Guo (22); Ado (72); m^2_2Guo (17); t^6Ado (18). Taken from Ref. 2 with permission.

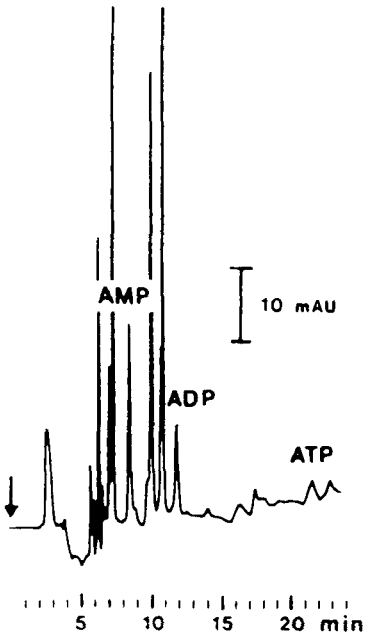

FIG. 5: Coupled-column analysis of adeninnucleotides in 500 µl human serum:
Precolumn: Phenylboronic acid modified polyvinyl-copolymer; Analytical Column: LiChrospher 100RP-18e, 5 µm, 250 x 4 mm I.D. **Loading:** 0.1 M Ammonium acetate, 0.1 M Magnesium chlorid, pH 9.8, 5 min at 0.3 ml/min; **Transfer:** 0.1 Citric acid, 10 mM Tetrabutylammonium hydrogen sulfate, pH 3.0, 5 min at 0.8 ml/min; **Separation:** 0.1 M Citric acid, 10 mM Tetrabutyl ammonium hydrogen sulfat, pH 3.0, MetOH-gradient from 0% to 30% in 28 minutes at 0.8 ml/min; Detection: UV at 260 nm; AMP = 1.3 nmol, ADP = 0.4 nmol, ATP = 0.1 nmol.

column (LiChrospher RP-18) by a column-switching device tolerates more than 4000 urin (100 µl) and more than 500 serum (500 µl) injections without any loss of its chromatography performance. The coupled-column technique allows the automated on-line sample clean-up and subsequent analytical resolution of ribonucleotides (e.g. AMP, ADP, ATP) or 17 different ribonucleosides and modified ribonucleosides under reversed phase conditions in less than 45 minutes. The recovery of the analytes after direct injection is quantitative, independent of the amount of analyte and independent of the biological fluid investigated.

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