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ON-LINE SAMPLE PROCESSING AND ANALYSIS OF DIOL COMPOUNDS IN BIOLOGICAL FLUIDS

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SUMMARY

We developed a coupled dual column system with an optional post-column derivatization for on-line sample processing, trace enrichment and analysis of aromatic 1,2-diol and aliphatic *cis*-diol biomolecules (*e.g.* catecholamines, ribonucleosides). The fully automated high-performance liquid chromatography analyzer tolerates the direct injection of proteinaceous fluids by use of a unique bonded-phase precolumn material which allows the simultaneous performance of covalent affinity and size-exclusion chromatography.

INTRODUCTION

Despite its high resolution power, sensitivity, precision and practicability, high-performance liquid chromatographic (HPLC) analysis of biological samples like blood, serum, plasma, urine, milk, lymphatic fluids, liquor, faeces or tissue homogenates is restricted by the pretreatment and processing of such highly complex matrices. Often the sample preparation represents the weakest chain segment in an analytical HPLC procedure as it still involves many elaborate, manual and thus error-prone and time consuming work-up steps. Thus, the goal in bioanalytical sample processing should be a rapid and if possible an automated, HPLC integrated procedure which exhibits a high selectivity for the analyte and which tolerates the direct injection of a biological sample. In many cases, analytes are present in too small amounts and biological samples are too complex or incompatible with conventional HPLC phase systems to permit an analysis by direct injection into an analytical column. Thus, simplification of such multicomponent mixtures as well as analyte enrichment is needed prior to analysis. In general, this is achieved by prefractionation or class separation and preconcentration steps. For this purpose the classical liquid–liquid extraction methods are increasingly substituted by liquid–solid extrac-

tion procedures, which use silica- or polymer-bonded phase materials packed in disposable cartridges or small, conventional columns. This successfully applied strategy allows sample processing in a manual or semi-automatic off-line mode under low pressure conditions or in an on-line mode under HPLC conditions. The application of electrically or pneumatically driven column switching valves even leads to fully automated on-line sample processing with subsequent analysis. The selectivity for a given analyte can be further enhanced by combining the pre-column technology with a post-column reaction system. Such a total system approach was demanded by Frei[1] and has recently been reviewed by Westerlund[2] for the direct injection of biological fluids, like plasma.

The most serious problem, however, encountered in the development of such bioanalytical HPLC systems is the quantitative removal of the protein matrix, as residual proteins limit the lifetime of the precolumn or deteriorate the efficiency of the analytical column.

Size-exclusion chromatography (SEC) is a very mild, effective and simple method for the quantitative removal of proteins, and unwanted sample constituents usually do not contaminate such columns even when used repetitively.

Thus, our strategy for on-line sample processing of proteinaceous fluids is the use of polymer-based size-exclusion materials as stationary supports. By additional chemical modification of these precolumn materials, we try to introduce the selectivity needed for extraction and trace enrichment of the analytes of interest.

The selectivity of a given material is determined by the different affinities of the sorbent with respect to the compound of interest and the residual matrix constituents. This is expressed by the different distribution coefficients or capacity factors, k', of the analyte *versus* a chemically bonded phase and a mobile phase. Dependent on the relative interaction energies, in the corresponding phase, the analyte will be retained at the stationary phase of the precolumn or move with the mobile phase. Taking into account the relative energies of the different interactions, *e.g.*, ionic, hydrogen bonding, dipole–dipole, Van der Waals, the ideal interaction with the bonded phase would be a covalent one. A prerequisite for such an ideal sample processing, however, would be only a temporary covalent fixation. This means that a total retention ($k' > 10^3$) of the analyte is followed by a total elution ($k' < 10^{-3}$). This "all-or-nothing" or "on-off" principle for extractive processing represents a special kind of affinity chromatography and can be described as covalent or "digital" chromatography.

In this paper two examples are given for on-line sample processing, trace enrichment and analysis of aromatic 1,2-diol and aliphatic *cis*-diol biomolecules (catecholamines, ribonucleosides) by a fully automated, commercially available HPLC system.

EXPERIMENTAL

Detailed conditions have been described for catecholamine analysis[3] and for ribonucleoside analysis[4–6].

Instrumentation
The HPLC analyzer comprises modular units from E. Merck (Darmstadt, F.R.G.) (Fig. 1): an LC controller Model L 5000 (LC-C); two pumps Model 655A-12

(P_1, P_2); an autosampler Model 655A-40 (AS); an automatic valve switching system Model ELV 7000 (AVS); a fluorescence detector Model F-1100 (FD) for catechol-amine analysis; an UV detector Model 655A-22 (UV) for ribonucleoside analysis; an integrator Model D 2000 (I); a precolumn (PC) and an analytical column (AC). For the optional use of post-column derivatization of the catecholamines norepinephrine and epinephrine to the corresponding trihydroxyindole derivatives, a reaction system Model 655A-13 (RS) was integrated.

Sampling

For catecholamine analysis, native human urine was acidified to pH 3.0–3.5 with 10 M hydrochloric acid and frozen at $-20°C$. 24-h Urine was collected in a receiver filled with 20 ml hydrochloric acid (25%), adjusted to pH 3.0–3.5 with 10 M sodium hydroxide and frozen at $-20°C$. The frozen urine samples were slowly thawed, centrifuged for 5 min in an Eppendorf desk centrifuge and 1 ml of the supernatant was transferred to an autosampler vial.

For plasma analysis of norepinephrine and epinephrine, 20 μl of a preservative solution (0.16 M EGTA, 0.3 M reduced glutathione, adjusted to pH 7.0 with sodium hydroxide), and for serum analysis 10 μl of a preservative solution (0.3 M reduced glutathione, adjusted to pH 7.0 with sodium hydroxide), were added per ml of freshly
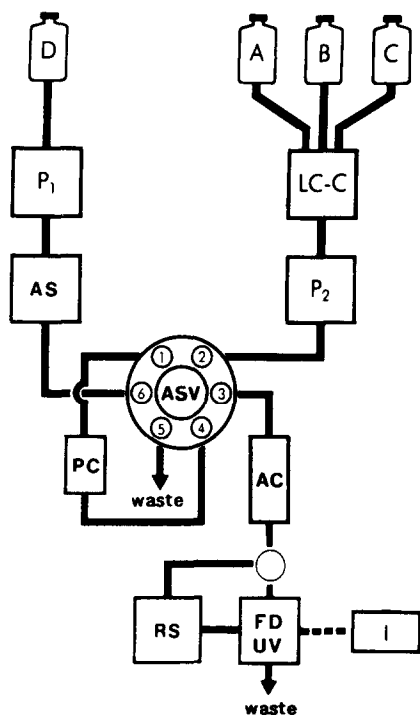

Fig. 1. Apparatus flow-diagram. LC-C = Liquid chromatograph controller; P_1, P_2 = pumps; AS = autosampler;ASV = automatic switching valve; PC = precolumn; AC = analytical column; FD = fluorescence detector; UV = ultraviolet detector; RS = reaction system; I = integrator; A = analytical buffer; B = methanol; C = doubly distilled water; D = precolumn buffer.

withdrawn blood sample. After the preparation of the plasma or serum fraction by centrifugation, the samples were stored at $-20°C$. These sampling procedures guarantee stability of the biogenic amines for at least 12 h at 4°C on the autosampler tray.

For ribonucleoside analysis in urine, serum and milk, samples were adjusted to pH 4.0 with concentrated formic acid and stored at $-20°C$ until investigation. Prior to analysis, thawed samples were centrifuged at 3000 g for 3 min and an aliquot (100 μl urine, milk; 500 μl serum) was applied to the HPLC system.

RESULTS AND DISCUSSION

Figs. 2 and 3 show the biomolecules investigated (unmodified, modified and hypermodified ribonucleosides as well as the parent catecholamines), the quantitation of which in body fluids is of importance in pathobiochemistry and clinical chemistry as these compounds serve as diagnostic marker molecules for a variety of distinct metabolic disorders.

For on-line sample processing, *i.e.*, covalent affinity chromatography the aromatic 1,2-diol and the aliphatic *cis*-diol compounds were chosen as a selectivity
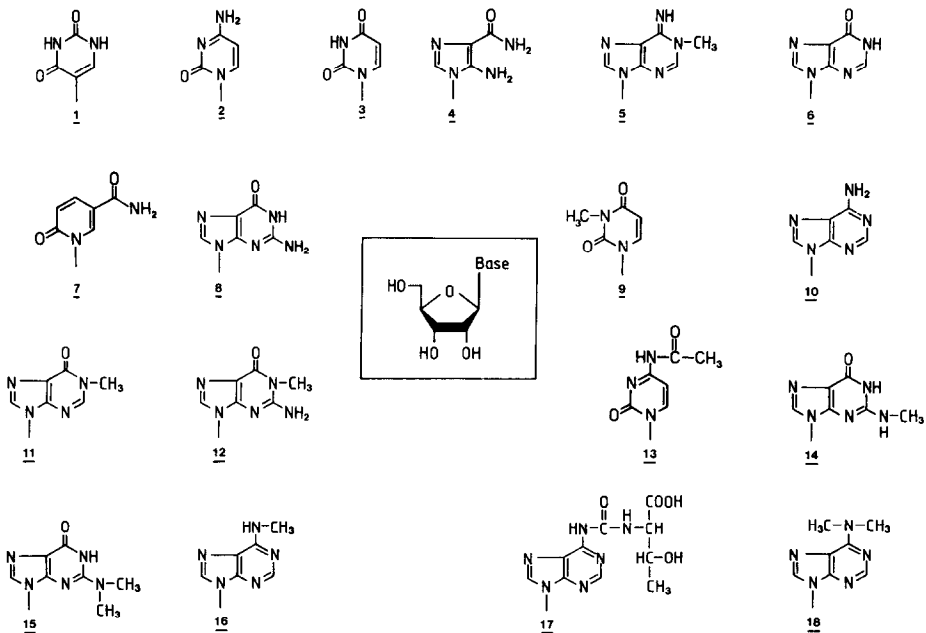

Fig. 2. Structural formulae of ribonucleosides and catecholamines investigated. 1 = Pseudouridine (ψ); 2 = cytidine (Cyd); 3 = uridine (Urd); 4 = 5-aminoimidazole-4-carboxamido-N-ribofuranoside (AICAR); 5 = N^1-methyladenosine (m^1 Ado); 6 = inosine (Ino); 7 = 2-pyridone-5-carboxamino-N-ribofuranoside (PCNR) = 1,6-dihydro-6-oxo-1-(β-D-ribofuranosyl)-3-pyridinecarboxylic amide; 8 = guanosine (Guo); 9 = N^3-methyluridine (m^3 Urd); 10 = adenosine (Ado); 11 = N^1-methylinosine (m^1 Ino); 12 = N^1-methylguanosine (m^1 Guo); 13 = N^4-acetylcytidine (ac^4 Cyd); 14 = N^2-methylguanosine (m^2 Guo); 15 = N^2-dimethylguanosine (m_2^2 Guo); 16 = N^6-methyladenosine (m^6 Ado); 17 = N-carbamoyl-threonyadenosine (t^6 Ado); 18 = N^6-dimethyladenosine (m_2^6 Ado).
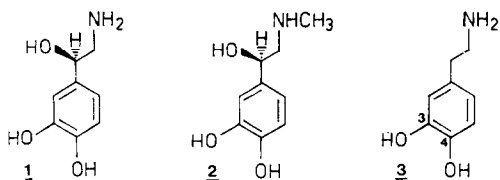
Fig. 3. Structural formulae of catecholamines investigated. 1 = R-(−)-1-(3,4-Dihydroxyphenyl)-2-aminoethanol (norepinephrine, NE); 2 = R-(−)-1-(3,4-dihydroxyphenyl)-2-(methylamino)ethanol (epinephrine, E); 3 = 2-(3,4-dihydroxyphenyl)ethylamine (dopamine, DA).

criterion, as these structural moieties reversibly form a cyclic diester with tetrahedral boronic acid under alkaline conditions[7]. This affinity ligand has been immobilized via its 3-aminophenyl derivative to various gel supports, e.g., agarose, cellulose, polyacrylamide, and used for the manual or partially automated[8] clean-up of ribonucleosides[9–18] and catecholamines[19] in an off-line mode. For on-line analysis of catecholamines in urine, the use of silica-based boronic acid affinity supports in coupled-column HPLC systems has been described[20–22]. For direct injection and on-line processing of plasma samples, Edlund and Westerlund[22] developed a liquid chromatographic system comprised of three coupled columns, two of which are precolumns for selective isolation (phenylboronic acid modified polyacrylamide) and subsequent enrichment (silica RP C_{18}). In contrast to the system described here, this method requires time consuming reconditioning steps for the additional enrichment column, complex, partially laboratory-made instrumentation and, due to the low recovery, the addition of an internal standard.

An analogous approach, i.e., a pH-dependent on-off mechanism for catecholamine sample processing, was studied by Frei[1] in a model system based on ligand-exchange chromatography on a copper(II)-loaded iminodiacetate-modified silica.
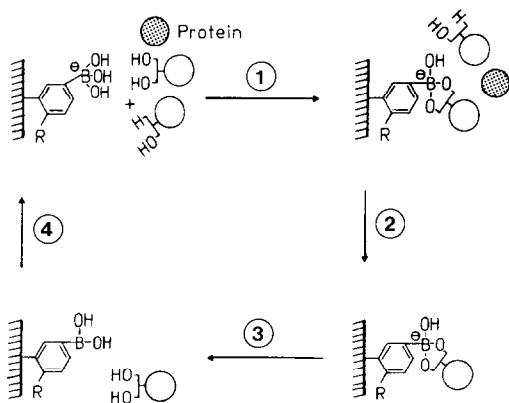

Fig. 4. Chromatographic properties of the sample processing precolumn. (1) pH-dependent covalent affinity chromatography of aromatic 1,2-diol and aliphatic cis-diol compounds, pH > 8.0; (2) SEC of residual matrix constituents; (3) on-line transfer elution (pH 3.0) of diol compounds to analytical column; (4) pH-controlled regeneration of precolumn.

For direct injection and on-line processing of ribonucleosides in urine, we developed a coupled dual-column system using a laboratory prepared phenylboronic acid modified silica[23] as precolumn packing material[24–27]. By preparing a new bonded-phase material[28] we succeeded in the development of the first dual-column HPLC analyzer for ribonucleosides[4–6] and catecholamines[3] in proteinaceous body fluids.

Fig. 4 schematically shows the chromatographic properties of the precolumn material. The stationary phase is a chemically modified co-polymer which tolerates pH values from 1 to 12 and a back pressure up to 2000 p.s.i.

The column material allows the simultaneous performance of two different modes of liquid chromatography. First, by virtue of its size-exclusion properties, residual matrix constituents, *e.g.*, proteins can be separated quantitatively from the solution[3]. Secondly, by immobilizing a specifically modified phenylboronic acid to the gel support, high-performance affinity chromatography (HPAC) can be performed. The lifetime of the precolumn material exceeds more than 3000 urine analyses (100-μl aliquots) and more than 500 plasma (500-μl aliquots) or milk analyses (100-μl aliquots) without reduction of the chromatographic efficiency.

In principle, the desired group-selective prefractionation, *i.e.*, on-line sample processing of ribonucleosides and catecholamines is carried out by a simple pH-step elution, followed by the analytical resolution under reversed-phase conditions. The overall on-line analysis cycle is characterized by five discrete steps:

(1) Sample application (10–500 μl) via the autosampler (*cf.*, Fig. 1). Chemoselective binding as well as enrichment of the diol compounds on the affinity ligand of the precolumn (*cf.*, Fig. 4) under slightly alkaline, *i.e.*, buffer D conditions ("HPAC step").

(2) Simultaneous, quantitative elution of the residual matrix constituents from the precolumn into the waste ("SEC step"; *cf.*, Fig. 4).

(3) Microprocessor controlled valve switching (*cf.*, Fig. 1). Quantitative, group-selective elution of the diol compounds from the precolumn (*cf.*, Fig. 4) by acidification (buffer A) of the immobilized cyclic boronate ester and simultaneous on-line transfer in a single, narrow elution band through positions 2, 1, 4, 3 of the valve (*cf.*, Fig. 1) to the top of the series-connected analytical column ("transfer step"; *cf.*, Fig. 4).

(4) Automated valve switching (*cf.*, Fig. 1) and separation of the diol compounds on the analytical column under reversed-phase chromatographic conditions ("separation step"; *cf.*, Fig. 4).

(5) Reconditioning of the tetrahedral trihydroxyboronyl functionality (*cf.*, Fig. 4) for a new extraction cycle during the analytical step with the initial eluent D ("regeneration step").

Figs. 5–11 represent typical chromatograms, which demonstrate that the compounds investigated can easily be identified and quantitated using the analytical approach described.

For method validation, a comparison of on-line with purely analytical column (off-line) chromatography of standard mixtures was performed. It revealed correlation coefficients between 0.96 and 1.00 for ribonucleosides and catecholamines, respectively. Fig. 12 shows typical chromatograms obtained for catecholamine analysis with natural fluorescence detection.
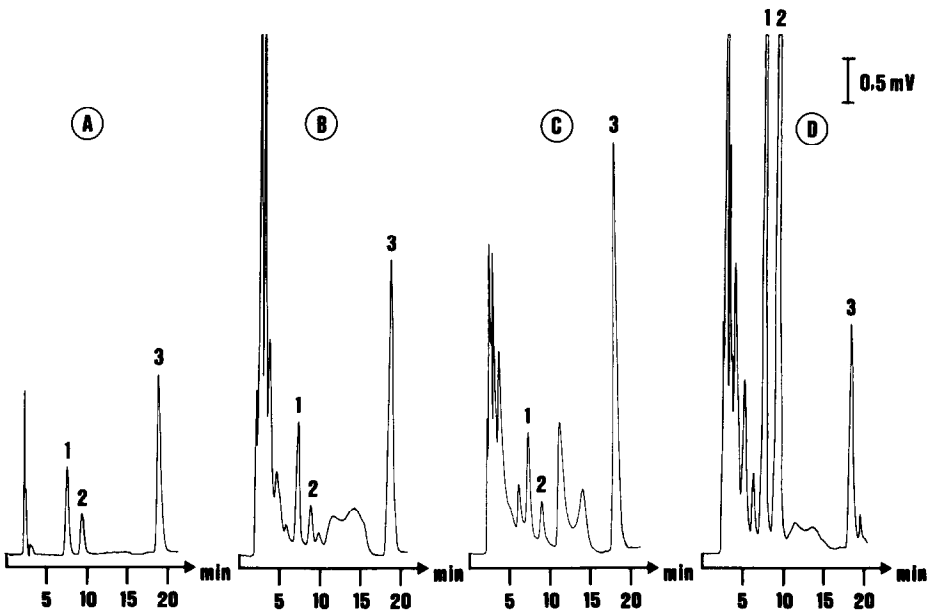
Fig. 5. Automated on-line analysis of free urinary catecholamines with natural fluorescence detection. (A) Standard (pmol), sample volume 100 μl: 1 = norepinephrine, 50; 2 = epinephrine, 20; 3 = dopamine, 130. (B) Native urine (pmol), sample volume 100 μl: 1, 60; 2, 20; 3, 250. (C) 24-h urine (pmol), sample volume 100 μl: 1, 60; 2, 20; 3, 340. (D) Pathological urine (phaeochromocytoma) (pmol), sample volume 100 μl: 1, 260; 2, 290; 3, 160.
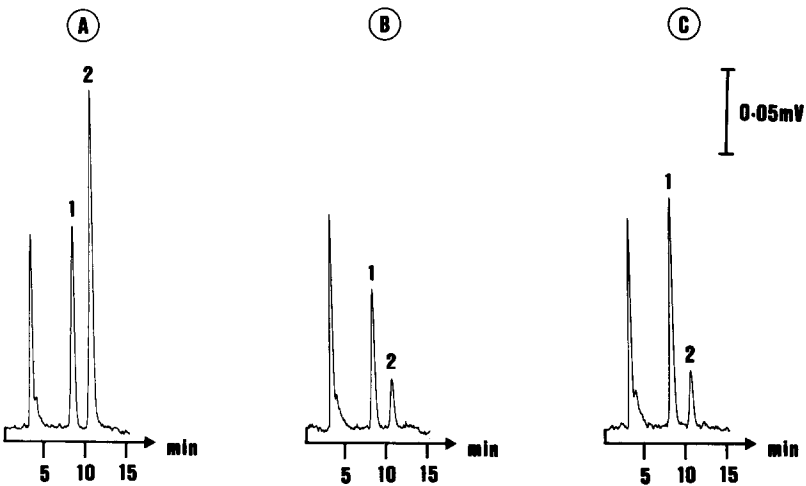
Fig. 6. Automated on-line analysis of free catecholamines in plasma and serum with trihydroxyindole fluorescence detection. (A) Standard (pmol), sample volume 100 μl: 1 = norepinephrine, 3.4; 2 = epinephrine, 2.8. (B) Plasma (pmol), sample volume 500 μl: 1, 2.4; 2, 0.3. (C) Serum (pmol), sample volume 500 μl: 1, 3.6; 2, 0.3.
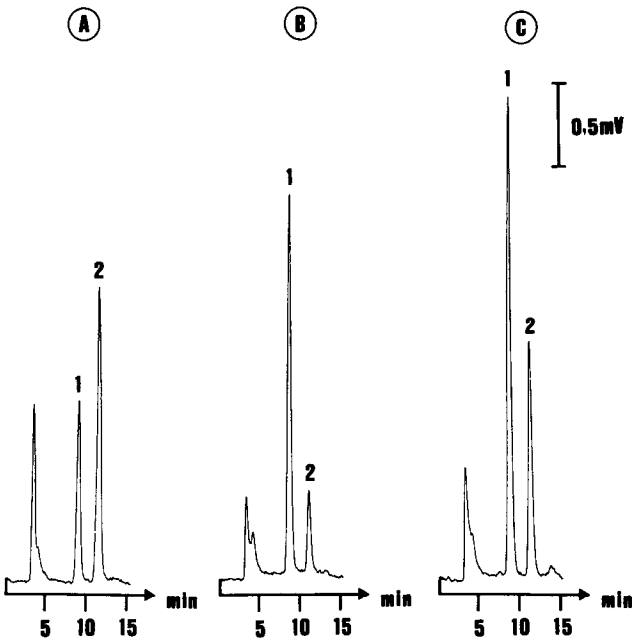
Fig. 7. Automated on-line analysis of free urinary catecholamines with trihydroxyindole fluorescence detection. (A) Standard (pmol), sample volume 200 μl: 1 = norepinephrine, 6.4; 2 = epinephrine, 5.6. (B) 24-h urine (pmol), sample volume 50 μl: 1, 13.6; 2, 1.3. (C) Native urine (pmol), sample volume 50 μl: 1, 17.6; 2, 3.9.
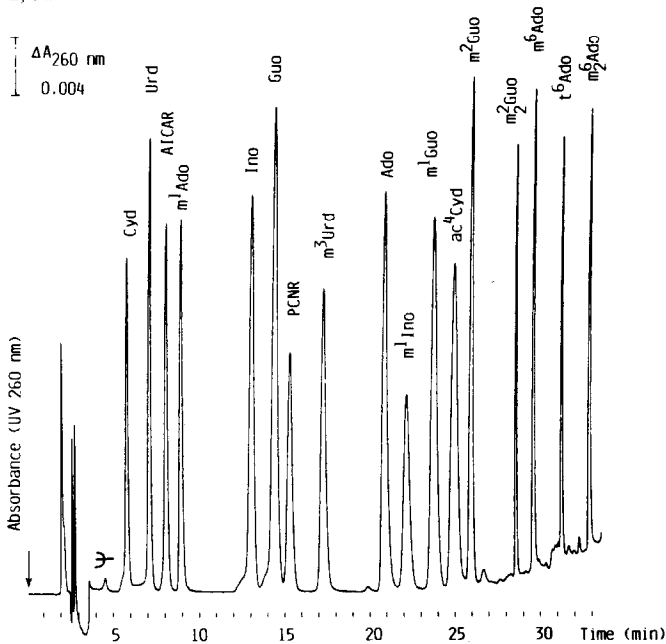

Fig. 8. Automated on-line analysis of a synthetic mixture of ribonucleosides. Sample volume: 100 μl. Compounds (nmol): AICAR (0.35); m^1 Ado (0.81); Ino (2.06); Guo (1.83); PCNR (1.49); Ado (1.30); m^1 Ino (1.88); m^1 Guo (2.11); ac^4 Cyd (1.05); m^2 Guo (1.03); m^2_2 Guo (0.47); m^6 Ado (0.54); t^6 Ado (0.53); m^6_2 Ado (0.62).
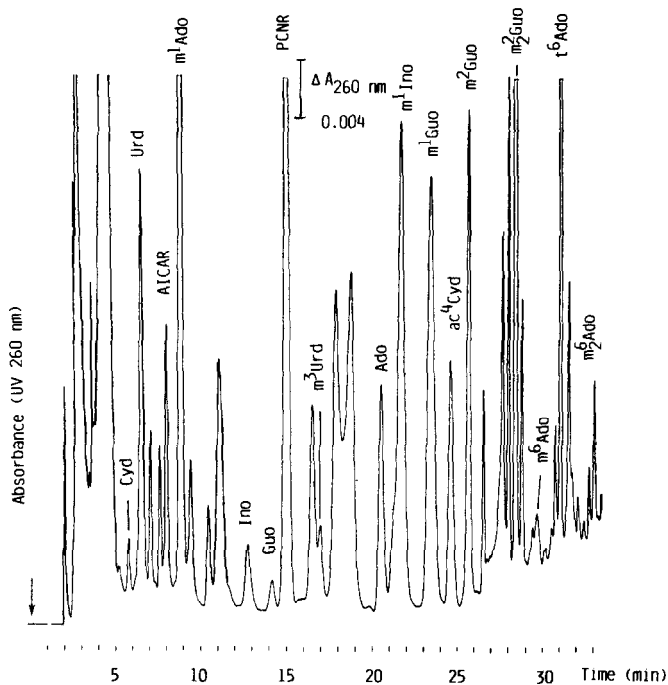
Fig. 9. Automated on-line analysis of ribonucleosides in 100 μl normal human urine. Compounds (nmol): AICAR (0.27); m^1 Ado (4.17); Ino (0.42); Guo (0.09); PCNR (4.87); Ado (0.71); m^1 Ino (5.43); m^1 Guo (2.88); ac^4 Cyd (0.59); m^2 Guo (1.10); m^2_2 Guo (4.21); t^6 Ado (1.98); m^6_2 Ado (0.09).
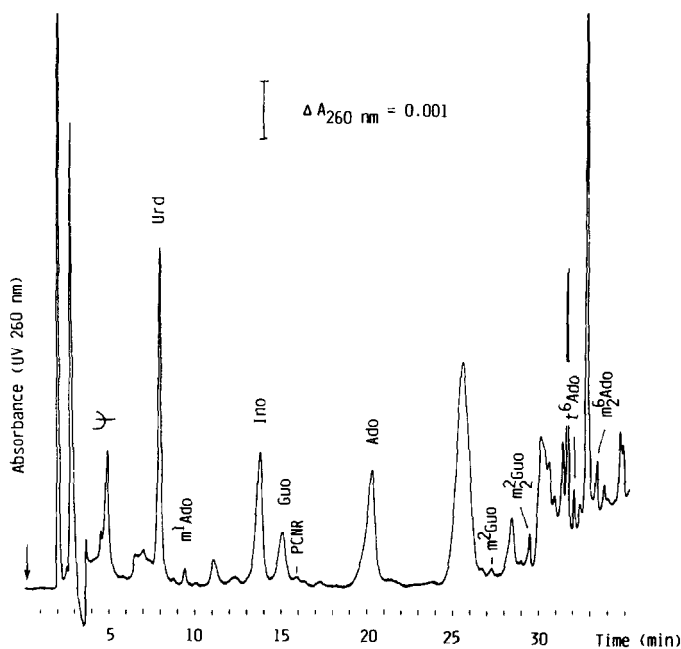

Fig.10. Automated on-line analysis of ribonucleosides in 500 μl normal human serum. Compounds (pmol): m^1 Ado (6); Ino (199); Guo (72); PCNR (17); Ado (174); m^2 Guo (7); m^2_2 Guo (14); t^6 Ado (57); m^6_2 Ado (31).
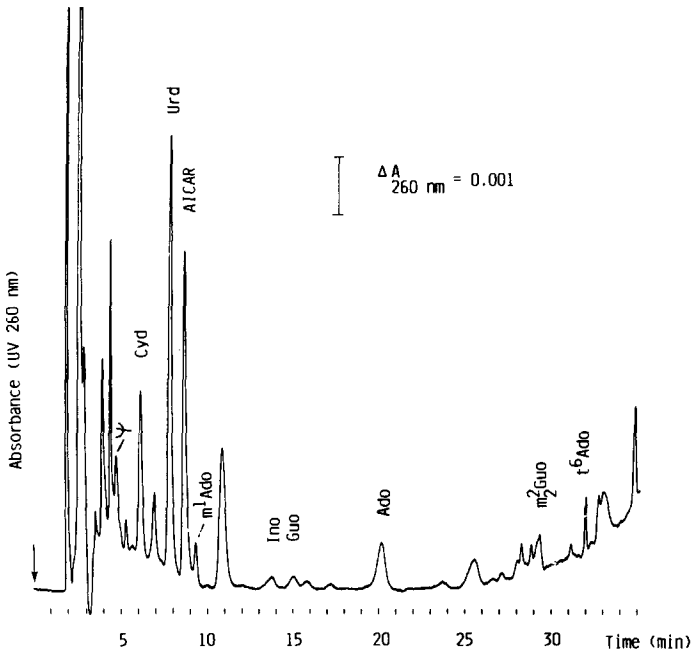
Fig. 11. Automated on-line analysis of ribonucleosides in 100 μl breast milk. Compounds (pmol); AICAR (84); m^1 Ado (32); Ino (35); Guo (22); Ado (72); m_2^2 Guo (17); t^6 Ado (18).
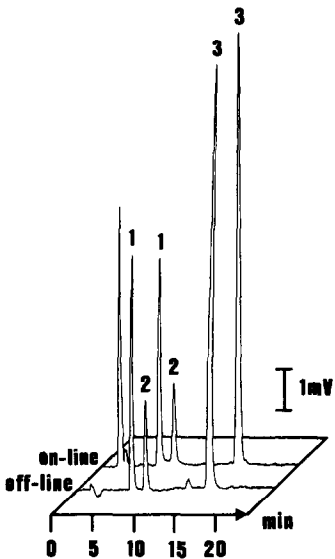

Fig. 12. Comparison between a catecholamine analysis of a standard sample with physiological concentrations in the off-line and the on-line mode. (sample volume 100 μl). 1 = Norepinephrine, 50; 2 = epinephrine, 20; 3 = dopamine, 210 pmol.

TABLE I

RELIABILITY

	Catecholamines	Ribonucleosides
Recovery	96–103%	95–104%
Imprecision*	3.9–6.3%	2.7–5.6%
Inaccuracy*	0.1 pmol (3.7%)	n.d.
Specificity	Chemoselective	Chemoselective
Detection limit**	20 fmol	10 pmol
Linearity	50 fmol–12 pmol***	0.25–25 nmol
	100 pmol–3 nmol§	

     * For physiological concentrations and day-to-day analysis.
    ** For 1-ml sample volume; $\underline{x} = x + 3\sigma$ where $\underline{x}$ = detection unit, x = mean value of peak area of blank sample matrix at the appropriate retention times, and σ = standard deviation.
    *** Trihydroxyindole fluorescence.
    § Natural fluorescence.

    The recovery of the analytes after direct sample injection is quantitative, independent of the amount of analytes and independent of the biological matrix investigated. Thus, this method does not require the addition of an internal standard and calibration can be performed simply with reference samples. The data in Table I document the reliability of the coupled dual column system.

CONCLUSIONS

    The use of a coupled dual column system for on-line sample processing, trace enrichment and analysis of aromatic 1,2-diol and aliphatic cis-diol biomolecules (catecholamines, ribonucleosides) has led to the development of a fully automated HPLC analyzer which tolerates the direct and repetitive injection of proteinaceous body fluids. The precolumn technology is based on a newly developed and unique bonded-phase material which allows the simultaneous performance of covalent affinity and size-exclusion chromatography.

    The high accuracy, based primarily on the quantitative and matrix-independent recovery of the analytes investigated, the practicability and the commercial availability of the HPLC analyzer render the system attractive for analytical investigations in the biochemical research field as well as in clinical laboratories.

    Further applications of this method will be: (1) trace enrichment for structural characterization of diol-containing compounds in biological fluids; (2) small scale preparation of natural diol-containing compounds; (3) investigation of disorders in catecholamine, ribonucleoside, ribonucleotide and/or RNA metabolism; (4) a non-invasive screening test (urinary modified ribonucleosides) for cancer diseases in humans; (5) investigation of renal reutilization processes; (6) therapeutic drug monitoring during nucleoside or catecholamine chemotherapy; (7) protocols for sample processing and on-line analysis of glycosylated proteins, e.g., haemoglobin A_1, coenzymes, e.g., reduced nicotinamide–adenine dinucleotides, ribonucleotides, e.g., ATP and dinucleotides, e.g., Ap_4A.

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