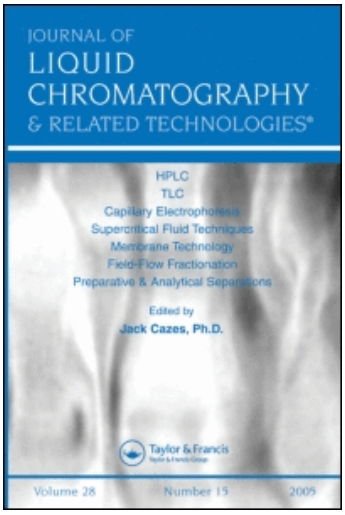

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DEVELOPMENT OF AN HPLC/DIODE ARRAY DETECTOR METHOD FOR THE DETERMINATION OF HUMAN PLASMA ADENOSINE CONCENTRATIONS

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ABSTRACT

Assays of adenosine and its derivatives in biological fluids involve fluorimetric, radioimmunological or chromatographic analyses. Techniques currently used are tedious because they involve either an extraction using immunological methods or one or two freeze drying cycles when using high performance liquid chromatography (HPLC). In this context, we describe a "quick" HPLC method using a diode array detector for the spectral analysis and quantitation of adenosine and its derivatives in less than two hours following sampling. We compared our results to those obtained with an HPLC technique coupled to UV detection.

Although there is a good correlation between the two techniques, the "quick" method provide values that are on average 30% higher than those obtained with the classical method. The absence of a lyophilization step in the classical method could explain this difference. The utilization of an HPLC system coupled to a diode array detector leads to a rapid and reliable assay of adenosine and its derivatives. The possibility of having a rapid assay method available could be very useful in cases of certain cardiovascular or neurological disorders.

INTRODUCTION

Adenosine is a nucleoside that has been studied in several physiological systems, including cardiovascular,[1] nervous[2] or immune system,[3] and the kidney apparatus.[4] Most adenosine arises from the dephosphorylation of ATP and then of AMP by a 5'nucleotidase. Adenosine is then either converted back to AMP by a kinase or deaminated to inosine by an adenosine deaminase; the final adenosine degradation pathway yields uric acid.[5] The half life of adenosine in the bloodstream is very short, of the order of several seconds.[6,7] This rapid half life results from the very rapid transformation of adenosine to inosine by the adenosine deaminase and also from the very rapid uptake of the substance by red blood cells[8] by a transporter that has been recently identified.[9]

Several sampling and analysis techniques have been reported for the study of adenosine metabolism. The aim of sampling techniques is to assure the ultra-rapid mixing of the blood sample and a blocking agent (stopping solution), containing inhibitors of intra-erythrocyte transport and inhibitors of adenosine deaminase. Thus, dypiridamole is a competitive inhibitor of cell membrane transport,[10-12] while ectonucleotidase activity present on the surface membrane of blood cells is inhibited by the divalent cation chelating agent EDTA.[13] 5'nucleotidase is inhibited by (α,β methylene adenosine 5' diphosphate (AOPCP).[14] Finally, EHNA (erythro-9(2-hydroxy-3 nonyl)adenine and 2'deoxycorformycin are potent inhibitors of adenosine deaminase.[15,16]

The sampling methods using the ultra-rapid mixing of blood and stopping solution are composed of a double syringe[17] or a double lumen catheter.[18] We have recently described a sampling technique using a vacuum tube for the simultaneous aspiration of blood and the stopping solution, and their rapid mixing.[19] Once the already delicate sampling phase has terminated, several quantitative assay methods for adenosine and its metabolites have been described, including fluorimetric assay,[20] radioimmunoassay[21,22] or HPLC.[23-29] These techniques are long and tedious since they require either extraction followed by radioimmunoanalysis[22] or require after deproteinization at least two

freeze-drying cycles before HPLC identification and quantitation.[17,19,28] The methods proposed until the present time have not led to a quantitation that is both reliable and rapid. The aim of the present work was thus to develop a chromatographic analysis method for nucleosides that would combine simplicity, rapidity, and reproducibility, and to compare the results obtained with those provided by a more conventional HPLC method.

EXPERIMENTAL

Adenosine Assay

Adenosine (crystallized, 99% pure), adenosine deaminase (calf intestine, specific activity 200 IU/mg) and dipyridamidole (5 mg/mL) were obtained from Boehringer Mannheim (France), inosine (99% pure), (α,β-methylene-adenosine-5'-diphosphate (AOPCP) and deoxycoformycin were obtained from Sigma. 9-erythro (2-hydroxy-3 nonyl) adenine (EHNA) was obtained from Burroughs Welcome. Heparin (25 UI/mL) was obtained from Choay laboratory. Econo-column (100 x 3 mm) and anion exchange resin (AG I -X2 acetate) were obtained from Bio-Rad (Richmond CA, USA). The reversed phase chromatography columns (Merck LIChrospher C 18, 250 x 4 mm. and RP 8 150X4 mm) were obtained from Merck, France. Methanol and other reagents were obtained from Merck, France.

Blood Samples

All samples were collected from 12 healthy volunteers (seven women and five men between 19 and 50 age, mean 37) as follows: total venous blood (3 mL) was drawn simultaneously with a stopping solution in Vacutainer tubes under vacuum (see Figure 1). This method enabled the blood sample to be rapidly mixed with 7 mL of stopping solution, which prevents adenosine uptake by red cells.[19] The stopping solution was composed of 0.2 mM dipyridamole; 4.2 mM Na2 EDTA; 5 MM EHNA; 79 mM AOPCP; heparin sulfate, l UI/mL; 1μM deoxycoformycin; 0.9% NaCl.

The correct mixed solution was controlled by measuring hematocrit of the samples (Mean values of hematocrit 45+/-3%; Mean values of the hematocrit with stopping solution 15 +/-5%). The sample plus stopping solution was centrifuged at 1500 g for 10 min, and the supernatant was deproteinized by adding 1 mL of 6 N perchloric acid for 1 mL of plasma, before a second centrifugation (3500 g for 10 minutes).

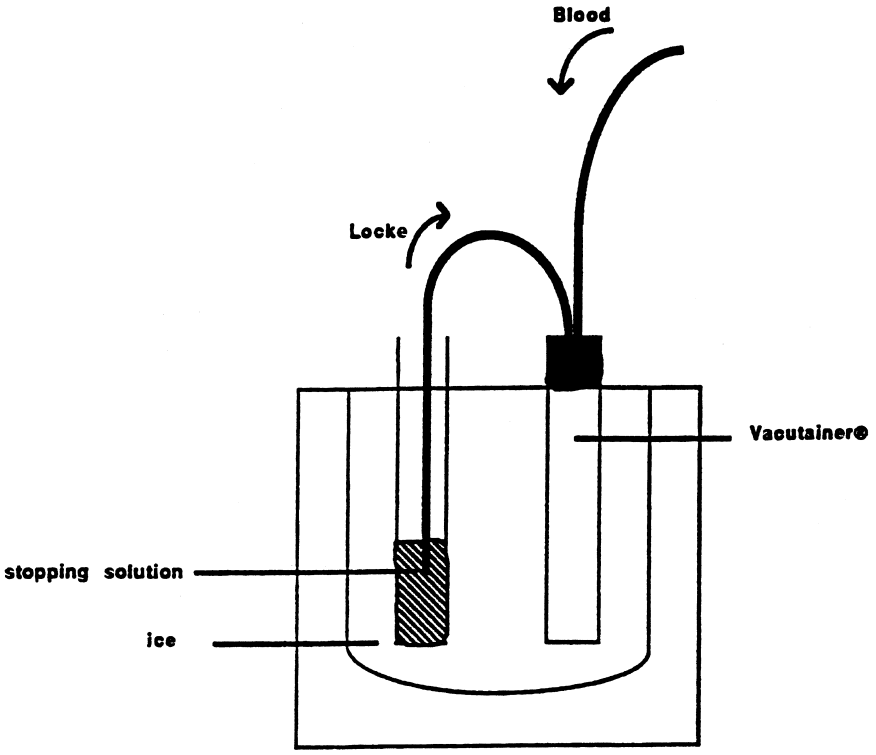

Figure 1. Sample system for rapid mixing of the blood sample and stopping solution. This solution contains a mixture of adenosine deaminase inhibitors and inhibitors of erythrocyte uptake systems (see material and methods). Rapid mixing is obtained by the simultaneous aspiration of venous blood and the stopping solution in a vacuum tube at the temperature of melted ice.

Conventional HPLC

Samples

Deproteinized samples of 4 mL (containing 500 μL plasma + 1.5 locke solution + 2mL perchloric acid) were neutralized by passage through an AG1 -X2 anion exchange column. Samples (plasma + Locke solution) were loaded on a column (Econo-column, 100 x 10 mm) previously equilibrated with pure water, and eluted with 10 mL of 5 mM acetic acid, frozen, Iyophylized and then redissolved in I mL of 50 mM sodium phosphate buffer (NaH2PO4/Na2HP04; pH4). The resulting solutions were filtered by centrifuging in a Millipore Ultrafree-MC 0.45 mm filter before being chromatographed.

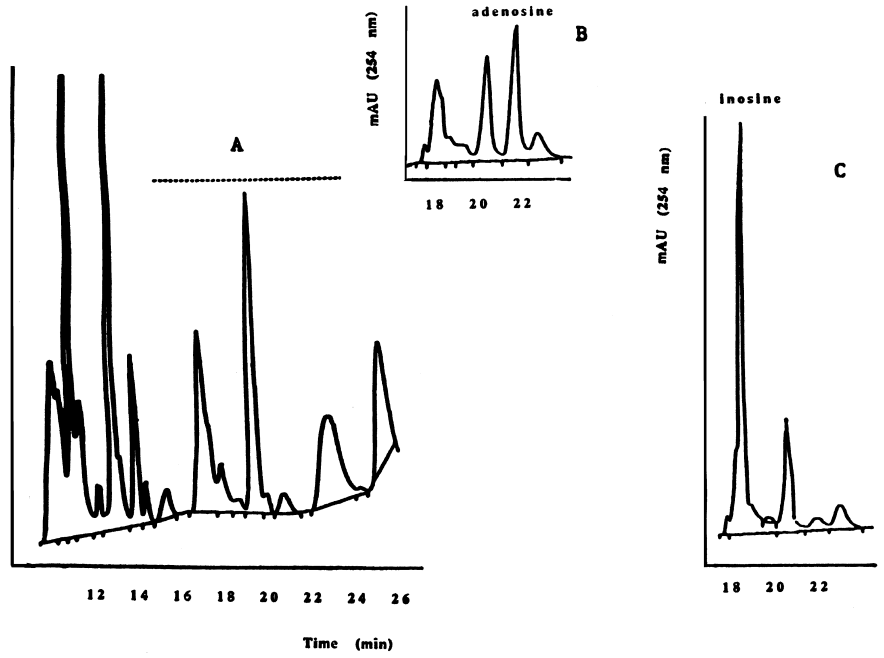

Figure 2. Chromatographic analysis of a plasma sample from an healthy volunteer. Deproteinized samples were neutralized by passage through an AGI-X2 anion exchange column, freeze dried (speed vac) and redissolved in I mL Phosphate buffer, before chromatography (see material and methods). The peaks eluting between 15 and 22 min were recovered (A) freeze dried and redissolved in 1-mL of phosphate buffer; 0.5 mL of sample were injected again into the column (B). The rest of the sample was incubated for 30 Min at 37°C with 10 IU of deaminase and injected in the column. The peak of adenosine disappears and the peak of inosine appears (C).

Equipment

Samples were analyzed chromatographically by high performance liquid chromatography (Kratos HPLC 4000) fitted with a 1 mL loop. Absorbance was measured at 254 nm and eluted peak areas were measured with a Shimatzu Chromatopac C-RCA integrator.

Chromatography

The technique has been previously described.[17,19,28] Briefly, plasma adenosine were assayed by reversed-phase HPLC on Merck C 18 columns (250 x 4 mm). The column was equilibrated with 50 mM sodium phosphate buffer

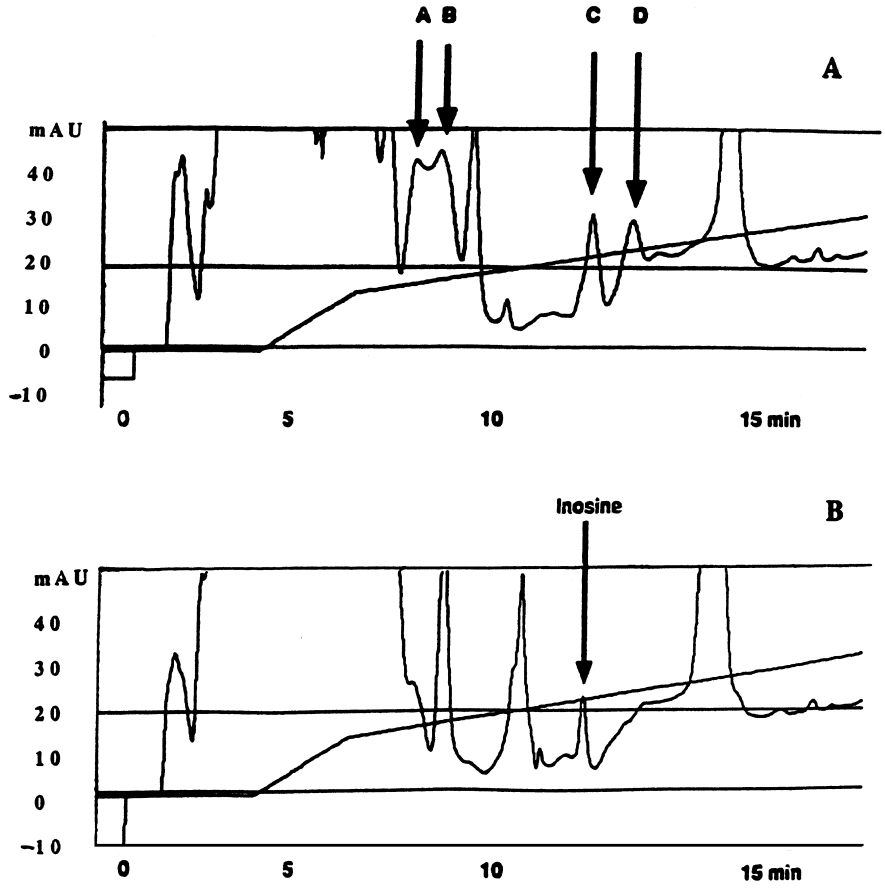

Figure 3. Chromatogram obtained after deproteinizing and filtering a sample of venous blood: 500 µL of venous blood deproteinized with an equal volume of 6 N perchloric acid were added to 500 µL of phosphate buffer (Na_2HPO_4/NaH_2PO_4), pH 4. A methanol gradient (gradient of methanol in green) and furnished a retention time of 11 min for inosine and 12.5 min for adenosine. A, B, C, and D were respectively hypoxanthine, xanthine, inosine and adenosine. In A, Adenosine concentration was 2.5 the detection threshold (5pM), and was without coelution with other metabolite. In B, chromatogram. of blank plasma, without adenosine, but with inosine.

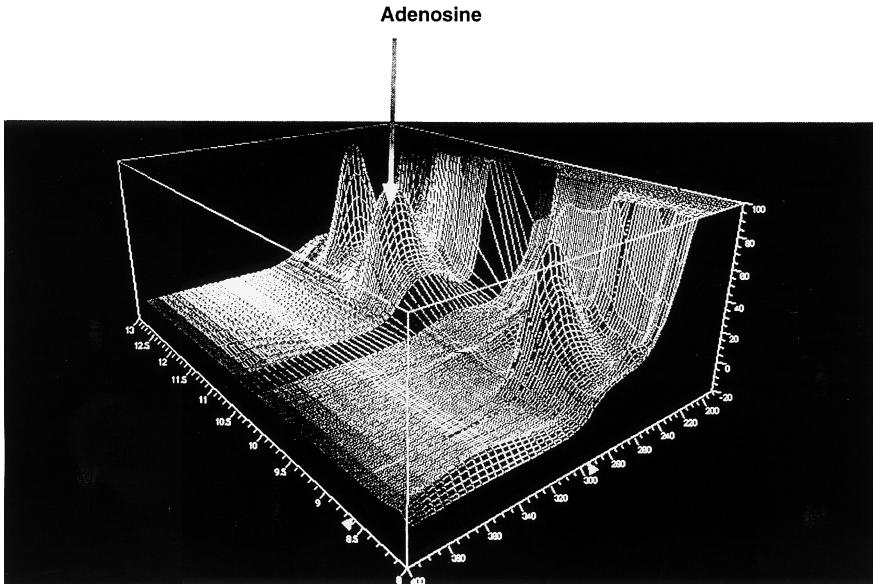

Figure 4. 3-D representation (time x absorption peak x mAbs units) of the peaks eluted according to the conditions described in Fig. 3. This representation enables partial coelution to be detected and to define the degree of peak purity.

(pH 4). Samples of 1 mL were injected and was eluted with a methanol gradient (0 to 46% methanol in 40 minutes) at a flow-rate of 1 mL/min. Adenosine was identified by elution time and by incubation with adenosine deaminase which increases the inosine peak and decreases the adenosine peak (see Figure 2). Adenosine was quantified by comparing the peak areas given by known concentrations of adenosine. In these conditions, sensitivity threshold was 10 pmol of adenosine standard in 1 mL injected (500 μL of plasma matrix + 500 μL of phosphates buffer). The intra-test coefficient of variation was 2%

Diode Array Detector HPLC

Equipment

A Hewlett Packard HP 1100 modular system was used, with a diode array detector (G13135A) and a deuterium lamp (slit 8 nm). The column used was 150 x 4 mm packed with RP8 (Merck) and a 1mL injection loop. The column was equilibrated as before with 50 mM phosphate buffer, pH 4.

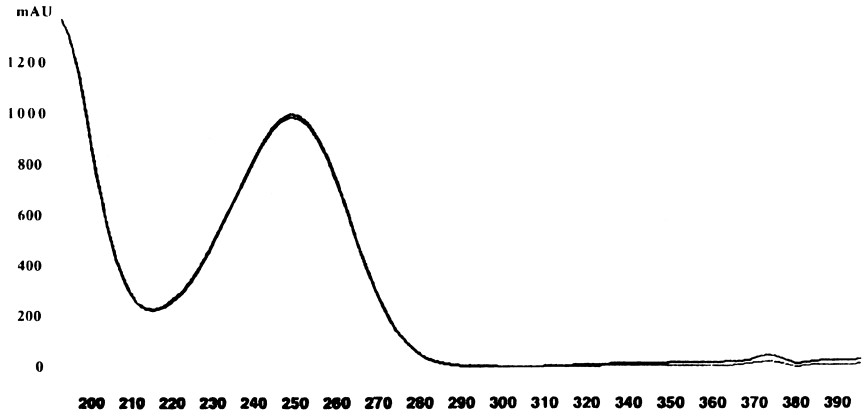

Figure 5. Comparison of spectra of an adenosine standard with the peak eluted at 12.5 min, in the chromatogram of a volunteer. Spectral comparison enables the peak eluted in the patient sample to be identified as adenosine.

Chromatography

0.5 mL of deproteinized plasma was mixed with 0.5 mL of phosphate buffer (pH 4) and injected in the column. Samples were eluted with a methanol gradient (see Figure 3 gradient of solvent B in green line). In these conditions, the elution time of standard adenosine was 12.5 min and that of inosine was 11 min (see Figure 3). The intra-test variation coefficient was 0.5% and the limit of detection of adenosine at 254 nm was 2 pmol in l mL injected (500 µL of plasma matrix + 500 µL of phosphates buffer). During chromatography, the spectra of peaks eluted between 8 and 15 min were recorded automatically at the rate of 6 spectra per second, in the 190 to 400 nm window, at 4 nm intervals.

Identification

The spectra were then compared to adenosine reference spectra (Figs. 4-6).

Quantitation

The calibration curve was plotted, from six points of 0, 0. 1, 0.25, 0.5, 0.75, and 1 nM of extracts standards in plasma matrix of nucleosides and metabolites. The calibration curve generated by these standards were used for quantitation of analytes in subject plasma samples.

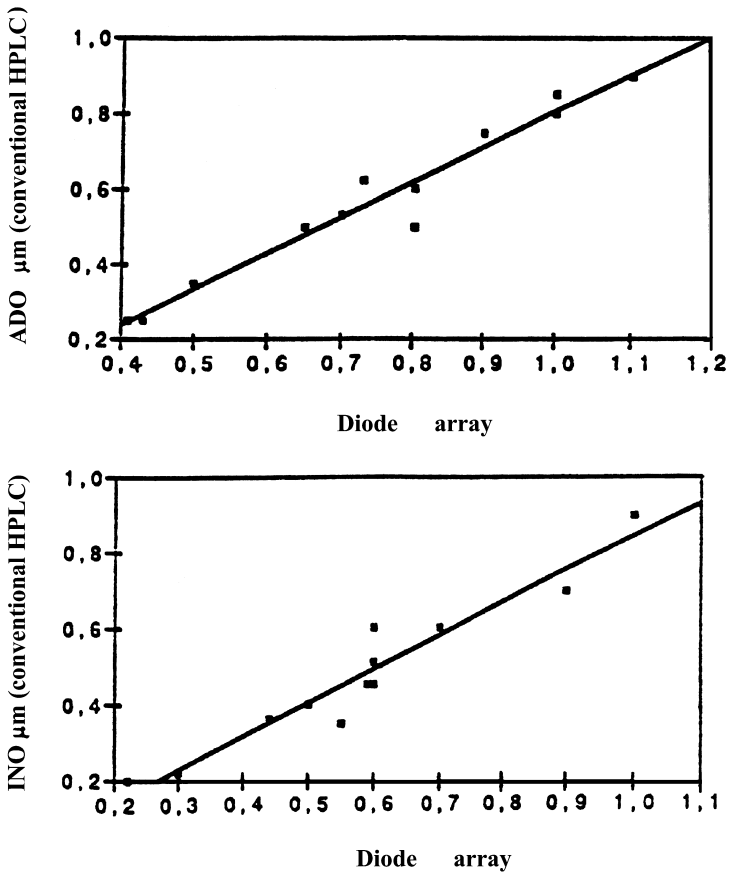

Figure 6. Correlation between the concentration of plasma adenosine (ADO) and inosine (INO) evaluated with the two HPLC techniques described. Spearman's r = 0.95, p = 0.0015, and r= 0.94, p= .003 respectively.

Statistical Analysis

Mann Whitney test and Spearman correlation coefficient was used to compare the results obtains with the two techniques.

RESULTS AND DISCUSSION

The concentrations of adenosine and its metabolites determined in 12 samples of blood from healthy volunteers were of the same order as published

Table 1

Individual Values (in μM), Means and Standard Deviations of Adenosine Plasma Levels and Derivatives, Evaluated with Two HPLC Techniques

UV Detector HPLC		Diode Array Detector HPLC			
Adenosine	**Inosine**	**Adenosine**	**Inosine**	**Hypoxanthine**	**Xanthine**
0.62	0.51	0.73	0.6	0.7	0.35
0.5	0.45	0.65	0.6	0.5	0.4
0.25	0.22	0.41	0.4	0.15	0.2
0.9	0.9	1.1	1.2	0.25	0.15
0.5	0.35	0.8	0.6	0.6	0.3
0.53	0.4	0.7	0.6	0.8	0.25
0.6	0.45	0.8	0.59	0.7	0.3
0.35	0.36	0.5	0.5	0.4	0.15
0.85	0.7	1	0.9	0.8	0.5
0.25	0.2	0.43	0.22	0.5	0.23
0.75	0.6	0.9	0.6	1	0.6
0.8	0.6	1	0.7	0.99	0.35
Mean 0.57	0.47	0.75	0.62	0.6	0.31
SD 0.22	0.22	0.22	0.24	0.25	0.13

values.[1,17,21] The nucleosides concentrations measured with the quick HPLC technique were significantly higher than those evaluated with the conventional technique (Adenosine:MannWhitney test; P=.048 ; inosine p= 0.04 and see Table 1). Nevertheless, the results obtained with both techniques were well correlated (Figure 6).

The sampling technique coupled with HPLC and diode array detection led to a reliable, reproducible, and rapid assay of adenosine and its metabolites. Quantities determined with the two HPLC methods showed that the correlation between the two techniques was good. Adenosine levels determined with the conventional method were on average 30% lower than with the rapid method. There are at least two possible explanations for these differences: first, a quantity of adenosine is probably bound to the resin in the Econo-column, during sample preparation with the conventional method; second, it is probable that the two freeze-drying cycles do not lead to the 100% recovery of nucleosides originally present in the samples. It is to be noted that although immunology techniques appear to be more sensitive[21,22] only HPLC enables both adenosine and its derivatives to be measured in the same sample.

The use of an efficient HPLC system coupled with a diode array detector leads to a rapid, reliable, and reproducible quantitative assay in samples from humans. Whereas classical methods often require several days and a large number of operations, the present technique can furnish results within several hours after drawing blood.

It would appear of value to have available a rapid assay method for adenosine and its derivatives in certain clinical situations. As an example, it has been shown that nucleoside levels in the coronary sinus are correlated with the chances for success of coronary angioplasty in myocardial ischemia.[28] Other indications involving the nervous system have also been mentioned, where the rapid determination of adenosine levels could indicate appropriate therapies.[29-31] It is highly probable that a large number of indications will be found in the coming years.

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