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Note

Determination of sixteen nucleotides, nucleosides and bases using high-performance liquid chromatography and its application to the study of purine metabolism in hearts for transplantation

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Nucleotide determination in human myocardial biopsies is a widely accepted tool in investigations concerning myocardial preservation during heart surgery [1,2]. Although the adenosine triphosphate (ATP) level is most important, concentrations of other nucleotides and nucleotide degradation products provide additional key information concerning the ability of the heart to recover from ischemia [3].

High-performance liquid chromatography (HPLC) is widely used for determination of nucleotides and related metabolites in the heart. Conventional ion-exchange methods only allow determination of the nucleotide concentration in the sample [4] such that nucleoside and base concentrations require separate determinations. Simultaneous separation of nucleotides, nucleosides and bases was achieved using reversed-phase octadecylsilica packing materials [5]. However, poor peak shape remained a problem even if the method was further modified by gradient elution [6,7]. Concurrently, reversed-phase ion-pairing methods were applied to analysis of purine metabolites in myocardial tissue. This chromatographic technique provides good peak shapes and per-

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mits separation of a wide spectrum of metabolites [8]. However, it requires special precautions during sample preparation [9].

The present report describes a modified reversed-phase procedure in which a fully end-capped, 3-μm octadecylsilica packing material has been utilised. This method allows the separation of all the major purine metabolites and is notable for improved peak shape, decreased run time and increased sensitivity in comparison with others. Data are presented here confirming the suitability of this method for studies of high-energy phosphate and purine metabolism in tissue from myocardial biopsies.

EXPERIMENTAL

Reagents and standards

HPLC-grade acetonitrile (S-grade) was obtained from Rathburn Chemicals (Walkerburn, U.K.). Fisons (Loughborough, U.K.) HPLC-grade potassium dihydrogen orthophosphate was obtained from the Southern Scientific Supplier (Harlow, U.K.). Aristar-grade potassium chloride was obtained from BDH (Poole, U.K.). Purine standards were all from Sigma (Poole, U.K.). HPLC-grade water was prepared using an Elgastat Spectrum purification system (Elga, High Wycombe, U.K.).

Instrumentation

The chromatographic system consisted of a Merck-Hitachi LiChrograph 6200 intelligent pump and a Merck-Hitachi LiChrograph L-4000 variable-wavelength UV detector (BDH Instruments, London, U.K.). The analytical column (150 mm$\times$4.6 mm I.D.) was packed with 3-μm Hypersil ODS (Hichrom, Reading, U.K.). A guard column (20 mm$\times$2 mm I.D.) packed with 10-μm Spherisorb ODS2 (Phase Separations, Queensferry, U.K.) was connected between the injector and the analytical column. Samples were introduced using a Rheodyne 7125 (Berkeley, CA, U.S.A.) injection valve equipped with a 20-μl loop. Sample peaks were integrated and quantified using a Turbochrom (Perkin-Elmer Nelson, Warrington, U.K.) chromatography data system operating on an IBM PS/2 Model 80 microcomputer.

Chromatographic conditions

Buffer A was 150 m*M* potassium dihydrogen orthophosphate solution, containing 150 m*M* potassium chloride adjusted to pH 6.0 with potassium hydroxide. Buffer B was a 15% (v/v) solution of acetonitrile in buffer A. The composition of the mobile phase was controlled by a low-pressure gradient mixing device. The amount of buffer B was changed linearly between the following time points: 0 min, 0% B; 0.1 min, 3% B; 3.5 min, 9% B; 5 min, 100% B; 7 min, 100% B; 7.1 min, 0% B. The re-equilibration time was 4.9 min resulting in a cycle time of 12 min between injections. The flow-rate was 0.9 ml/min. The

analytical column was maintained at a constant temperature in the range 17–19°C by means of a cooling coil carrying running mains water. A constant column temperature in this range was essential for both peak resolution and reproducibility of retention times. Peaks were monitored by absorption at 254 nm for routine analysis. Occasionally the separation was repeated at 280 nm to monitor peak purity.

Sample preparation

Biopsy specimens were collected from the apex of the heart using Tru-Cut biopsy needles (Travenol Labs., Deerfield, IL, U.S.A.). Specimens were immediately washed free of blood in an ice cold, isotonic buffer solution containing 155 m*M* ammonium chloride, 10 m*M* potassium bicarbonate and 0.1 m*M* disodium EDTA before freezing in liquid nitrogen (within 15 s from the time of collection). After freeze-drying over a period of 12 h, tissue was extracted with 0.3 ml of 0.6 *M* perchloric acid in 1.5-ml Eppendorf tubes fitted with pestles as described previously [10]. Extracts were then centrifuged (11 000 *g* for 5 min) in a cooled microfuge before collection and neutralisation of 0.25 ml of the supernatant with approximately 0.08 ml of 2 *M* potassium hydroxide. Aliquots of this extract were injected into the chromatograph.

Reproducibility and recovery studies

Reproducibility of the whole extraction and analytical procedure was investigated in non-ischemic human myocardial tissue. A series of biopsies were collected from a transplant recipient's heart just after removal and processed using the procedure described above. Analytical recovery studies were performed by extraction of the tissue with perchloric acid containing a known concentration (10 μM) of all standards. Recovery was calculated after subtraction of the endogenous purine contribution, found through analysis of a parallel series, and is expressed as a percentage of the quantity added.

RESULTS AND DISCUSSION

Optimisation of the separation

Fig. 1a is a chromatogram of standards at a concentration in the middle of the working range (about 500 pmol of each standard). The peak shape of polar nucleotides is significantly improved compared with reversed-phase methods previously described [5,7]. The use of 3-μm packing materials enabled a shorter (15 cm) column to be used without sacrificing resolution. Moreover, benefits of shorter run time and increased sensitivity were also obtained. Initially, a problem was encountered with the separation of ATP and inosine monophosphate (IMP). Inclusion of potassium chloride together with a lowering of the column temperature resulted in baseline separation of both compounds. A problem with the resolution of nicotinamide-adenine dinucleotide (NAD) and

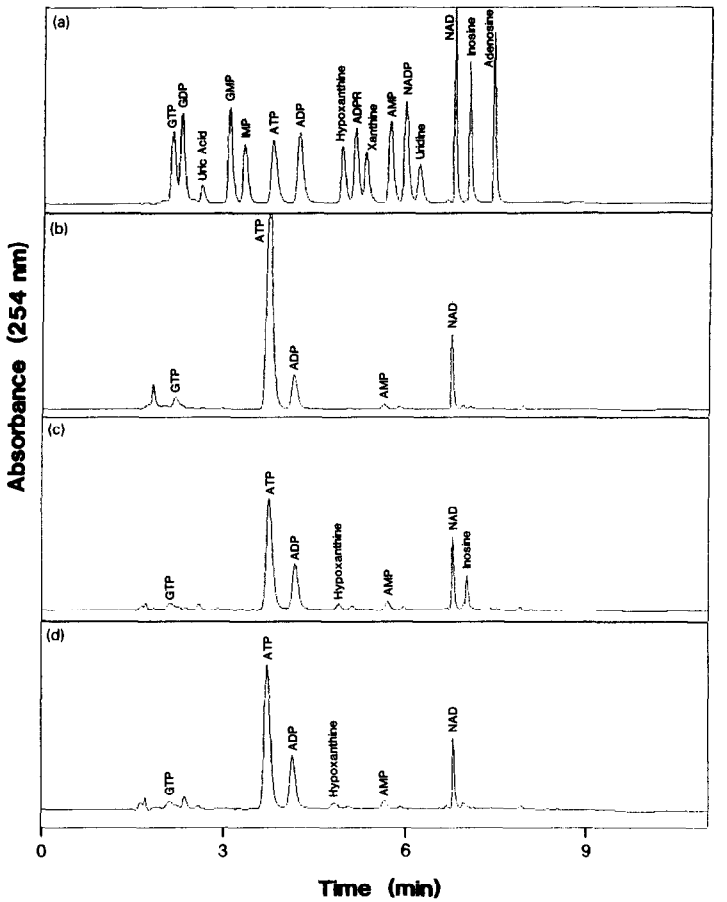

Fig. 1. Chromatograms of sixteen nucleotides, nucleosides and bases separated as described in the text. Abbreviations: AMP = adenosine monophosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; ADPR = adenosine diphosphoribose; GMP = guanosine monophosphate; GDP = guanosine diphosphate; GTP = guanosine triphosphate; IMP = inosine monophosphate; NAD = nicotinamide-adenine dinucleotide; NADP = nicotinamide-adenine dinucleotide phosphate. (a) Separation of sixteen standards; the amount of each compound injected was between 0.3 and 0.5 nmol. (b) Human myocardial biopsy collected from the apex of the donor heart before excision. (c) Biopsy collected from the donor heart before implantation. (d) Biopsy collected from the donor heart 30 min after reperfusion.

inosine was solved by appropriate adjustment of the gradient shape. NAD was induced to elute earlier than inosine by steepening the gradient curve. The calibration curves for the purine standards (peak area versus amount injected) were linear over the range 0–2000 pmol. Recovery of standards from perchloric acid extracts varied between 92 and 110%. The detection limit for most of the

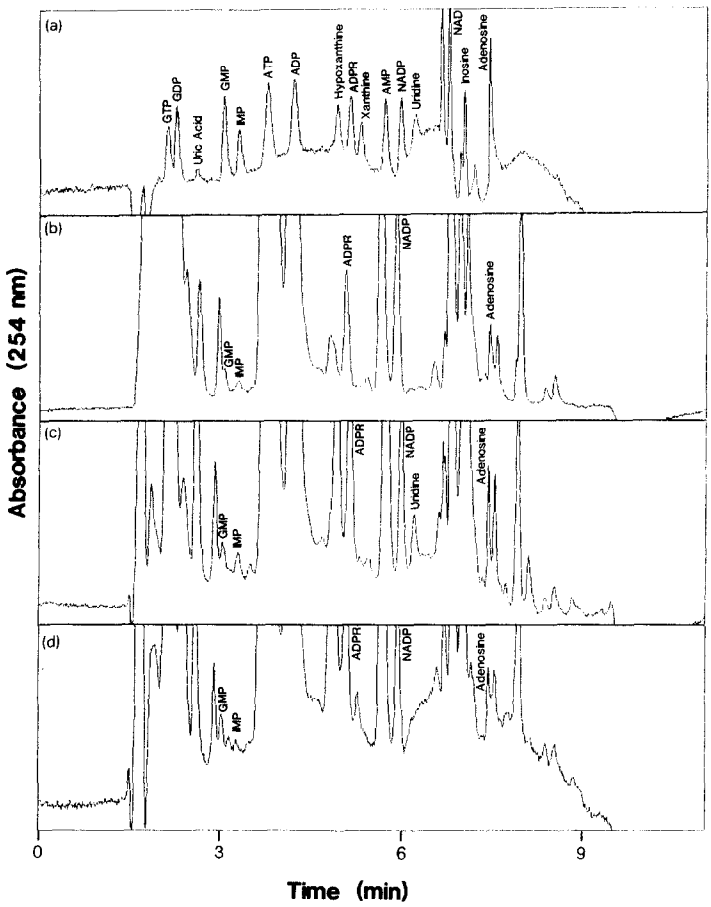

Fig. 2. Chromatograms at high sensitivity range. Details as in Fig. 1 except that the amount of standards injected in (a) was between 1.5 and 2.5 pmol.

compounds was approximately 1 pmol. The separation of standards at the limit of detection is presented in Fig. 2a.

Peak purity was established by replicate injections at different detection wavelengths. The 280 nm/254 nm absorbance ratios of standards and an extract of hypoxic human myocardial tissue were compared. An acceptable correlation was found for all the compounds investigated. However, the sensitivity of the method presented here is not sufficient to monitor the purity of the purine catabolites in non-ischemic tissue. Consequently, absolute values of these compounds cannot be determined. However, the method is capable of demonstrating an increase in their concentration.

The results of reproducibility studies are presented in Table I. In human myocardial tissue the coefficients of variation of compounds at a high concen-

TABLE I

COEFFICIENTS OF VARIATION (C.V.) FOR REPEATED ANALYSES IN THE SAME HEART

Multiple biopsies ($n=5$) were collected from the apex of the recipient heart 5 min after removal. See Fig. 1 for key to metabolite names.

Metabolite	Concentration (mean ± S.D.) (nmol/mg of protein)	C.V. (%)
ATP	24.1 ±2.0	8.3
ADP	8.51 ±0.72	8.5
AMP	2.07 ±0.36	17.4
GTP	1.01 ±0.28	27.7
GDP	0.35 ±0.10	28.6
GMP	0.066 ±0.018	27.3
IMP	0.069 ±0.027	39.1
NAD	3.84 ±0.14	3.7
NADP	0.18 ±0.02	11.1
ADPR	0.44 ±0.09	20.5
Adenosine	0.15 ±0.03	20.0
Inosine	0.81 ±0.12	14.8
Hypoxanthine	0.31 ±0.06	19.4
Uridine	0.12 ±0.02	16.7

tration, namely ATP, adenosine diphosphate (ADP) and NAD were the lowest, falling in the range 4–9%. Coefficients of variation for the other detectable compounds which, except for adenosine monophosphate (AMP) and guanosine diphosphate (GTP), were all at a concentration of less than 1 nmol/mg of protein were between 11 and 39%.

Determination of purine metabolites in human myocardial tissue

Fig. 1 shows chromatograms of acid extract obtained from human myocardial biopsies collected before harvesting of the donor heart (b), before starting the implantation procedure (c) and 30 min after reperfusion (d). The same chromatograms at a higher sensitivity are presented in Fig. 2b–d. The second biopsy collected shows a decrease in the ATP/ADP ratio together with a marked increase in inosine concentration. A minor increase in the levels of adenosine, uridine and IMP were also observed.

The HPLC procedure described in this paper is able to separate the complete spectrum of purine metabolites found in myocardial extracts. The advantages of this procedure compared to previous methods are a short run time allowing sample injections each 12 min, improved peak shape and increased sensitivity. The procedure is suitable for purine analyses of myocardial biopsies enabling nucleotide degradative processes in the hypoxic human heart to be followed.

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