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OPTIMIZATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF NUCLEOSIDES AND THEIR CATABOLITES

APPLICATION TO CAT AND RABBIT HEART PERFUSATES

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SUMMARY

A high-performance liquid chromatographic method is described for the separation of nucleosides and related compounds in a single isocratic run. The separation of a standard mixture of at least thirteen compounds is achieved within 15 min on a new type of reversed-phase column 25 cm long, filled with 5-μm particles of Select B. Pre-treatment of the silica particles before silanization has given this type of reversed-phase material unique characteristics for basic and acidic compounds. Chromatograms are shown to compare the effectiveness of the Select B column for the separation of nucleosides with that of other C_{18} and C_8 phases. The separation is achieved with 0.3 *M* ammonium dihydrogen phosphate (pH 4.00) and a mixture of methanol, acetonitrile and tetrahydrofuran. Detection is carried out with a variable-wavelength UV detector at 254 nm. Sample preparation and the influence of the organic solvents, pH and buffer concentration are described.

To illustrate the applicability of the method, representative chromatograms are shown of perfusates of Langendorff preparations and the working hearts of cats and rabbits. Remarkable differences were obtained before and after ischaemia and before and after treatment with a nucleoside transport inhibitor.

Baseline separation of cytosine, orotic acid, cytidine, uracil, uric acid, hypoxanthine, xanthine, inosine, guanosine, xanthosine, allopurinol, thymine and adenosine was achieved. The detection limit for these compounds was less than 1 ng per injection.

INTRODUCTION

The determination of nucleosides in biological fluids requires very sensitive and specific methods because of their low concentration and the presence of a variety of compounds. High-performance liquid chromatography (HPLC) provides many advantages, especially because of the capability of high resolution with indirect identification and of precise quantitation by an extremely sensitive UV detector with a convenient data system. A particular advantage of liquid chromatography is the

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possibility of analysing the unchanged biological material, in contrast to gas chromatography, where volatile derivatives are required. Many procedures have been used for the analysis of nucleosides and bases by HPLC[1,2]. Their separation can be achieved by chromatography in ion-exchange systems (static or dynamic)[3–7], reversed-phase systems[8–23] or even on unmodified silica[24].

In a previously described procedure[8], nucleotides and nucleosides were determined at the nanomole level with gradient elution. This method has the advantage of separating a large number of purine and pyrimidine derivatives. However, it has the disadvantage of requiring a relatively long analysis time. The additional re-equilibration time after gradient elution increases the analysis time by 50%. The objective of this study was to adapt this procedure or to develop another HPLC procedure in order to separate as many nucleosides as possible in a single, isocratic development. The method must be sufficiently sensitive to permit the direct analysis of perfusates from ischaemic hearts under several experimental conditions.

EXPERIMENTAL

Apparatus

The HPLC instrument was a Model 5560 (Varian Aerograph, Walnut Creek, CA, U.S.A.) equipped with a microcomputer-controlled reciprocating single-piston pump system. The UV detector was a Varian UV-200 (with a flow cell of 4.5 μl and a path length of 4 mm). Loop-valve injection (10 or 20 μl) (Rheodyne, Berkeley, CA, U.S.A.) was used to introduce the sample. All chromatograms were monitored with a microcomputer data system (Varian Vista 402). The raw data were stored on floppy disks.

Chemicals

Nucleosides and analogues were obtained from Janssen Chimica (Beerse, Belgium) [uric acid (UA), cytidine (CYD), hypoxanthine (HYP), xanthosine (XAO), inosine (INO), guanosine (GUO), uridine (URD), allopurinol (ALLO), thymidine (THD), adenine (ADE), guanine (GUA) and creatinine (CREA)], Sigma (St. Louis, MO, U.S.A.) [xanthine (XAN), uracil (URA), orotic acid (OA), thymine (THY) and cytosine (CYT)] and Boehringer (Mannheim, F.R.G.) [adenosine (ADO)]. These standards were of the highest purity available and each gave a single peak. However, xanthosine contained an impurity, which was seen in some chromatograms.

Solvents and other chemicals were purchased from Merck (Darmstadt, F.R.G.). Acetonitrile, methanol and tetrahydrofuran were of Uvasol quality and phosphoric acid and ammonia solution were of Suprapur quality. Buffer solutions were preparated daily from 85% phosphoric acid and 25% ammonia solution. Water for the buffers was quartz-distilled and filtered through a 0.2-μm filter. Buffer solutions prepared in this way needed no further purification, in contrast to buffers prepared from ammonium phosphate, which required purification by passage through a low-bar preparative C_{18} column.

Columns

Reversed-phase (RP) columns were purchased from Merck. RP-18 and RP-8 were of the LiChroCart Superspher cartridge type. These narrow-bore stainless-steel

columns (25 cm × 4 mm I.D.) are filled with spherical particles of 4 μm, having a pore size of 100 Å. Their efficiency ranged from 100000 to 120000 theoretical plates per metre. RP Select B columns were of the Hibar LiChrosorb type. These columns are filled with irregular, modified RP-8 particles of 5 μm with a pore size of 60 Å. Their efficiency ranged from 50000 to 70000 theoretical plates per metre. This RP material has a specific selectivity for basic compounds, resulting in symmetrical peaks. A further advantage is that the peak maxima and peak shapes are independent of the amount injected. This property makes the RP Select B phases very desirable for biological samples, where concentrations can never be predicted.

New columns were purified by pumping through at least 250 ml of methanol–acetonitrile (50:50, v/v), followed by 50 ml of quartz-distilled water and finally 250 ml of the buffer solution with the appropriate concentration of organic solvents. The column lifetime under these conditions was *ca.* 250 injections, but could be doubled by reversing the columns at regular intervals.

Chromatographic conditions

At 254 nm the sensitivity of the UV detection was 0.01–0.002 a.u.f.s. at a time constant of 0.5 s and a spectral bandwidth of 5 nm. The flow-rate was 0.7 ml/min in all experiments and the working temperature was ambient (20 ± 2°C).

The final eluent composition for the applications was 0.3 *M* ammonium dihydrogen phosphate (pH 4.00)–acetonitrile–methanol–tetrahydrofuran (THF) (100:1:1:0.4, v/v). Throughout, 10 μl-aliquots of a standard mixture, containing eleven of the most important nucleosides and catabolites, were injected. The concentration of each was 10 nmoles/ml of nucleoside. To determine the detection limits, 5-μl aliquots were injected. The injection volumes were always 20 μl for biological samples.

The capacity factor, k', for each compound was determined from the retention time relative to that of an unretained compound (ethylammonium chloride, a solute that shows some UV absorbance at 254 nm).

Sample preparation

To avoid enzymatic degradation of the nucleosides, the heart effluents were stabilized by heating at 95°C for 4 min. This did not change the composition of the sample and allowed these samples to be stored in a freezer (below −20°C) until taken for analysis. No change in composition was observed, even after storage for 1 year.

RESULTS AND DISCUSSION

Basic method

A reversed-phase system with a C_{18} alkyl-bonded silica column was selected, based on the excellent results reported earlier[8]. The mobile phase of ammonium phosphate and modifier [acetonitrile–methanol (50:50, v/v)] was chosen because of the efficient separation, but there were some disadvantages and reasons to search for another less time-consuming and less critical procedure.

One disadvantage of gradient elution, certainly at extremely high detector sensitivities, is the long equilibration time (50% of the HPLC analysis time). A second disadvantage is that, after several injections of biological material, impurities retained

on the column may contaminate some minor components in the eluate. Other reasons for changing from gradient to isocratic elution were that no nucleotides would be expected in the experiments planned and the fact that a compound of major interest, hypoxanthine, was eluted as a broad, tailing peak, resulting in a less sensitive and less accurate determination. It would be desirable to use the same column and the same mobile phase in the development of an isocratic procedure. This would require only a small adjustment in the percentage of the modifier and be less time consuming. To overcome the peak tailing of hypoxanthine, other columns could be tested and the pH of the buffer could be changed. However, other eluites would then tail, and therefore the optimum pH of 6.00 was kept constant in the first experiments. In the original procedure, it was also found that a 50:50 mixture of acetonitrile and methanol as the modifier, together with a buffer concentration of 0.15 *M* at pH 6.00, was the best compromise between retention times and symmetrical peaks.

Effect of the organic solvents in the eluent

RP C_{18} phase. In Fig. 1 capacity ratios are plotted against the percentage of the modifier, acetonitrile–methanol (50:50, v/v), giving an overview of the effect of the modifier at constant pH (6.00) and constant molar concentration (0.15 *M*) of the buffer. It is clear that no modifier concentration gave complete separation. While the addition of 6% of modifier results in a favourable analysis time of 20 min, only two components, ADO and UA, are completely separated. A 2% addition, on the other hand, gives an extremely long analysis time (over 70 min) without separation of all components, URD and XAN being unresolved. The most efficient separation was

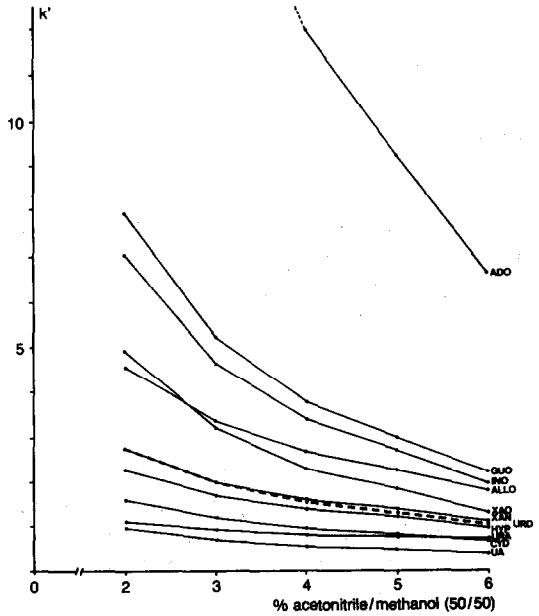

Fig. 1. Effect of the modifier [acetonitrile–methanol (50/50, v/v)] concentration in the mobile phase on the capacity factor, *k'*, of nucleosides and analogues. Stationary phase, LiChroCart Superspher C_{18}. Mobile phase, 0.15 *M* $NH_4H_2PO_4$ (pH 6.00).

obtained with a 4% modifier. Simultaneous elution of URD and XAN, the still relatively long retention time of ADO (> 30 min) and the wandering of URA, URD and XAO over the chromatogram all led us to decide to stop further development of isocratic chromatography on a C_{18} reversed-phase column.

RP C_8 phase. Although the high potential efficiency of this column (more than 120000 theoretical plates per metre for anthracene), the k' plot in Fig. 2 shows, in fact, that its efficiency is lower than that of the C_{18} columns. The shorter chain length of the alkyl-bonded phase results in a decrease of the retention times of all components. This decrease, together with the wandering of some compounds, is so dramatic that many compounds are unresolved.

RP Select B phase. As stated by the manufacturer, this stationary phase should have characteristics intermediate between those of the C_{18} and C_8 phases in combination with other selectivities. The effect of the modifier concentration on the k' values is plotted in Fig. 3. It is remarkable that the plots are nearly parallel, except for ADO. Two groups of two peaks are formed: URA + CYD and HYP + XAN. The total analysis time is acceptable for a 4% or higher concentration modifier.

Comparing the k' plots, it is evident that Select B can be selected for further development and optimization of the procedure in order to separate CYD + URA and HYP + XAN and to decrease the retention time of ADO.

Effect of pH

To study the effect of pH changes, the ionic strength of the eluent was held nearly constant at 0.15 *M*. The percentage of modifier was also held constant at 4%,

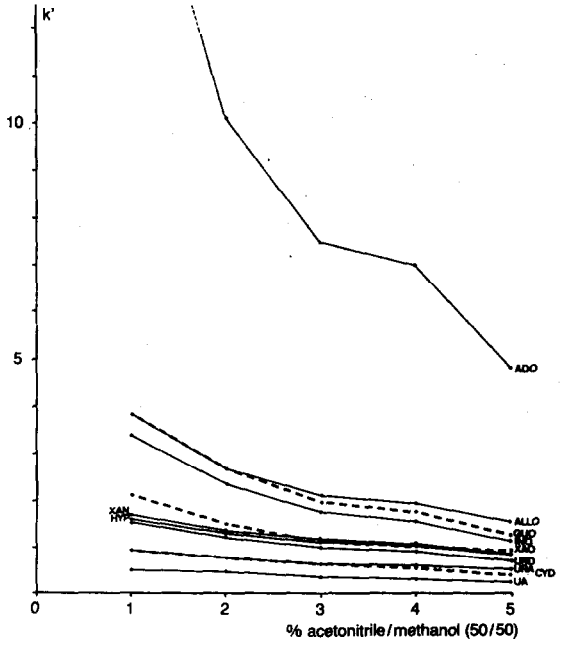

Fig. 2. Effect of the modifier concentration in the mobile phase on the k' value of nucleosides. Stationary phase, LiChroCart Superspher C_8. Other conditions as in Fig. 1.

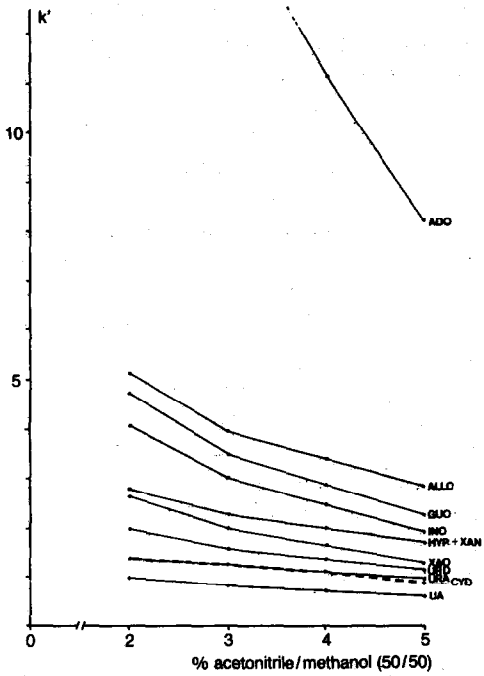

Fig. 3. Effect of the modifier concentration in the mobile phase on the k' value of nucleosides. Stationary phase, Hibar LiChrosorb Select B. Conditions as in Fig. 1.

an optimum determined by previous tests. In general, the retention behaviour of the nucleosides and their derivatives is in line with the classical concept of reversed-phase chromatography, in which charged species are rapidly eluted, whereas neutral molecules are retained on the hydrophobic packing. The effect of pH changes on the nucleosides and derivatives is shown in Fig. 4. The results are in complete agreement with those in the literature[7,14,15,22]. The retention behaviour of the nucleosides and derivatives is also as predicted from their pK_a values. URA, URD, HYP, XAN, INO, GUO and ALLO, which are largely neutral in the pH range covered, do not undergo a significant change in k' values with decreasing pH. However, for ADO and CYD, which are protonated at pH 2.2, the k' values are lower at pH 2.2 than at pH 6.00 where the compounds are neutral. The k' of CYD decreases only moderately when the pH of the eluent decreases from 6 to 3, whereas that of ADO decreases dramatically. However, XAO, with a pK_a of 5.7, shows a marked increase in k' when the pH decreases from 6 to 3. UA shows a moderate increase in retention with decreasing pH.

The optimum pH for the separation was 4.00. All the components are separated, except HYP and XAN, and the analysis is completed in 25 min. In contrast to other results[21] with the RP Select B stationary phase, almost no tailing was observed at higher pH.

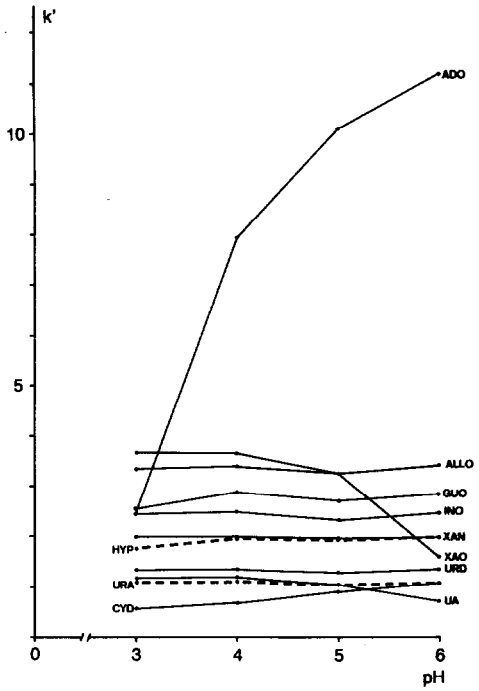

Fig. 4. Effect of pH of the mobile phase on the k' value of nucleosides. Stationary phase, RP Select B. Conditions as in Fig. 1 with 4% of modifier.

Effect of ionic strength

The ionic strength of the aqueous mobile phase at a constant pH of 4.00 was increased by increasing the higher buffer concentration (from 0.05 to 0.45 *M*). The influence of the ionic strength on the k' values is shown in Fig. 5. As can be observed, the ionic strength affects the retention behaviour of the nucleosides only minimally. This is in accordance with the literature[21]. Buffer concentrations between 0.05 and 0.25 *M* result in longer retention times for ADO. At a 0.3 *M* concentration, the k' values became more stabilized.

The optimal buffer concentration was 0.3 *M* with a 4% modifier content and at pH 4.00. A slightly better separation was achieved for URA and UA together with a remarkable decrease in the retention time of ADO (the analysis is completed in 18 min).

Optimization with THF

In order to retain the same order of the elution, the concentration of the modifier was decreased from 4 to 2%. As can be observed in Fig. 6, without THF, the k' values were increased and HYP and XAN were only partially resolved. The addition of small amounts of THF in 0.2% steps resulted in a decrease in the retention times and a baseline separation of all compounds in the test sample. Optimal separation was obtained with 0.4% of THF. Above 0.6% of THF, the k' values decreased dramatically, and with 1% of THF no resolution was obtained of the XAO–ALLO and INO–XAN pairs.

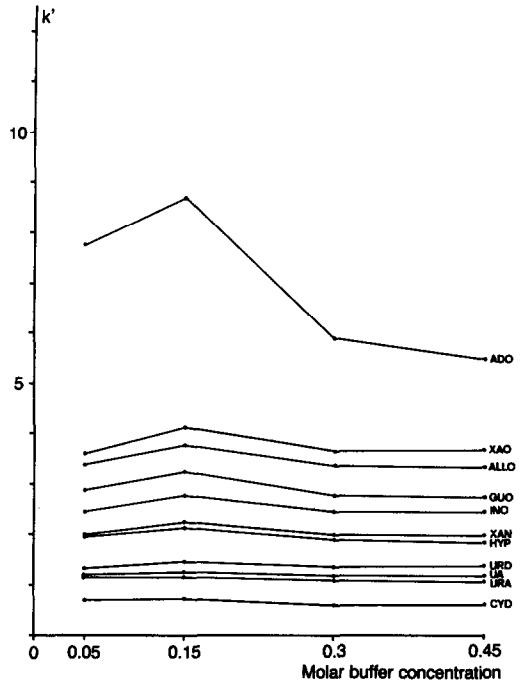

Fig. 5. Effect of the molar concentration of the mobile phase on the k' values at pH 4.00. Other conditions as in Fig. 4.

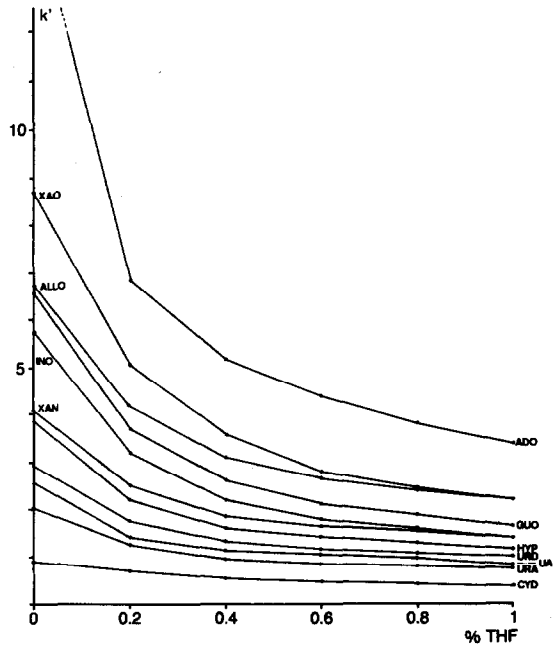

Fig. 6. Effect of THF on k' values. Column, Select B; mobile phase, 0.3 M buffer (pH 4.00) with 2% of organic modifier.

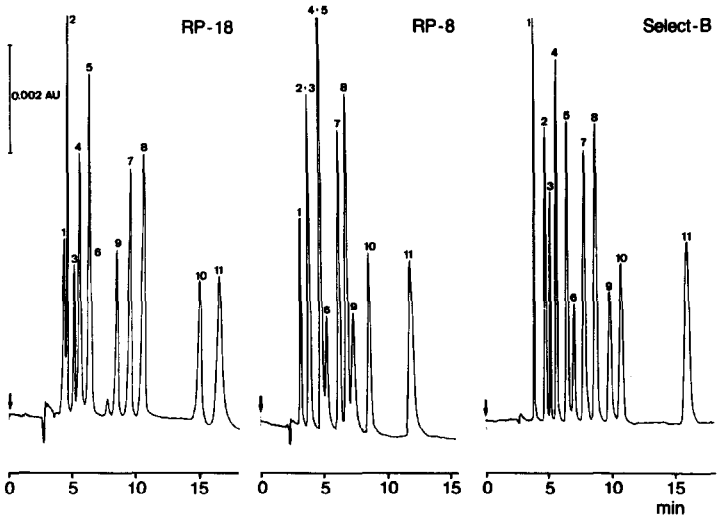

Fig. 7. Comparison of RP C_{18}, RP C_8 and Select B with optimal eluent: 0.3 *M* buffer (pH 4.00) containing 1% of acetonitrile, 1% of methanol and 0.4% of THF. Peaks: 1 = CYD; 2 = URA; 3 = UA; 4 = URO; 5 = HYP; 6 = XAN; 7 = INO; 8 = GUO; 9 = ALLO; 10 = XAO; 11 = ADO.

Fig. 7 is a chromatogram of a separation with 0.4% THF on RP Select B in comparison with the RP C_{18} and C_8 phases. All of the nucleosides of interest are completely separated. The analysis is completed in 17 min and no peak asymmetry is observed. The addition of THF to the mobile phase increases the theoretical plate number. It should be mentioned that the chromatogram in Fig. 7 was obtained after more than 300 injections.

Comparison of C_{18}, C_8 and Select B stationary phases

The best eluent for the separation of nucleosides and derivatives was 0.3 *M* ammonium dihydrogen phosphate (pH 4.00)–acetonitrile–methanol–tetrahydrofuran (100:1:1:0.4, v/v), at a flow-rate of 0.7 ml/min. Using this eluent, RP C_{18}, RP C_8 and RP Select B were compared (Fig. 7). It is obvious that only RP Select B gave a complete separation of the nucleosides. With RP C_{18}, ALLO and XAO wander over the chromatogram and HYP and XAN are unresolved. With RP C_8 the order of elution is not changed but more compounds are unresolved. On both RP C_{18} and C_8 peak tailing results in an asymmetric chromatogram.

Evaluation of the procedure

Peak identification in the perfusates was based on internal standards, retention times, absolute and differential UV absorption, time-of-flight UV spectra and enzymatic transformations. To ensure that no interference from other nucleosides and nucleobases can occur, a mixture of nucleobases and derivatives is injected using the final conditions. A compiled chromatogram was obtained on a new RP Select B column (Fig. 8). The dotted line represents the added compounds. A complete separation of the added from the other components was obtained for the CYT–OA pair. THY, GUA and ADE were fairly well separated, but there was no resolution of

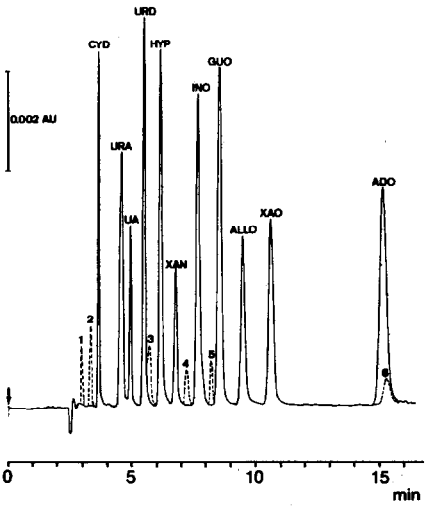

Fig. 8. Model chromatogram of nucleosides and analogues on Select B. Mobile phase, 0.3 *M* ammonium dihydrogen phosphate (pH 4.00)–acetonitrile–methanol–THF (100:1:1:0.4, v/v). Flow-rate, 0.7 ml/min; wavelength, 254 nm; 0.008 a.u.f.s. Peaks: 1 = CYT; 2 = OA; 3 = GUA; 4 = ADE; 5 = THY; 6 = THD.

ADO and THD. Only when the amounts of ADO were smaller than those of THD could separation be achieved. Using the described optimal procedure, a detection limit of less than 1 ng per compound was easily attained and all compounds were separated. However, CREA (not indicated on the chromatogram; no UV absorption at 254 nm) was eluted together with CYT.

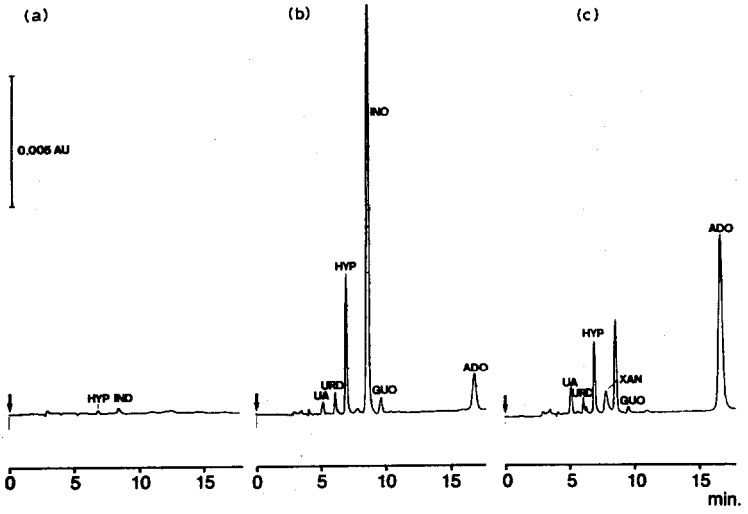

Fig. 9. Chromatograms of nucleosides in rabbit heart effluents on RP Select B, (a) during the control period, (b) 32 min of ischaemia, and (c) after the effect of mioflazine, a nucleoside transport inhibitor (NTI). Conditions as in Fig. 8.

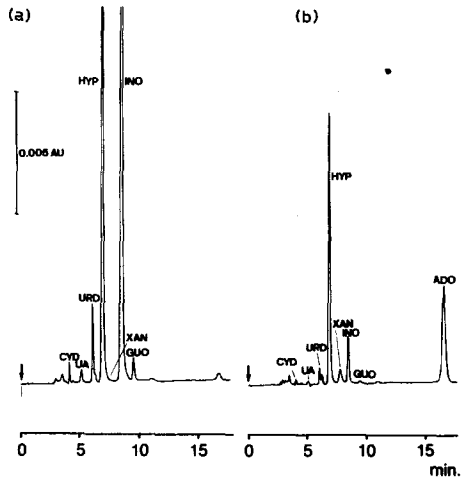

Fig. 10. Chromatograms of nucleosides in cat heart on RP Select B after 32 min of ischaemia, (b) with and (a) without mioflazine treatment. Conditions as in Fig. 8.

APPLICATION

Langendorff preparations

Isolated hearts were perfused at 37°C through the aorta with Krebs solution (+10 m*M* glucose), saturated with a mixture of oxygen–carbon dioxide (95:5). The constant-volume perfusion was carried out with a roller-pump at a flow-rate of 10 ml/min for rat and guinea pig and 25 ml/min for rabbit and cat hearts. The experimental procedure was as follows: 15 min normal perfusion, followed by *ca.* 30 min stopped flow, then 6-min perfusion. During ischaemia the hearts were kept at 37°C.

The perfusates were injected (20 μl) directly into the chromatograph or stored in a freezer prior to analysis.

Example

Chromatograms of heart perfusates from rabbit and cat are shown in Figs. 9 and 10. Differences in the release of nucleosides and their ratios and amounts were observed in the different species. XAN and UA were barely detectable in rabbit and cat perfusates. HYP is a major metabolite in the cat. The inclusion of a nucleoside transport inhibitor (NTI) had no effect on the effluent compostition in the rat, but a pronounced effect in cats and rabbits, where ADO became easily the major component. The significance of these findings is discussed elsewhere[25].

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