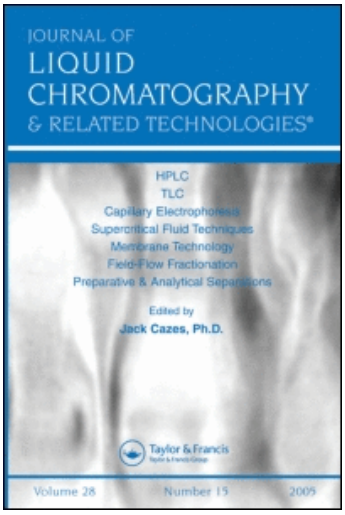

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THE ANALYSIS OF PURINES FROM RABBIT KIDNEY PRESERVATION MEDIA BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

This paper demonstrates the application of High Pressure Liquid Chromatography (H.P.L.C.) for the separation and measurement of purines from perfusates of rabbit kidneys preserved for transplantation. A μ-Bondapak C_{18} reverse phase column at pH 4.5 was included in a convex gradient elution program to facilitate optimal resolution in minimum time. The eluent comprised dipotassium hydrogen phosphate (K_2HPO_4) buffer with a final concentration of 10% methanol. A possible application of this method is discussed in relation to viability prediction of kidneys stored for transplantation.

INTRODUCTION

The increased practise of transplantation for treatment of acute renal failure has created a need for the successful preservation of kidneys. A considerable proportion of transplanted kidneys fail to support life due to irreparable damage incurred during storage. The need for an accurate viability test to eliminate those kidneys not capable of life supporting function after transplant has been generated.

Numerous tests have been suggested, none affording a definate correlation to viability. These include changes in vascular resistance (1,2), perfusion flow (1), weight gain (2,3), lactate levels (4-7), lactate dehydrogenase (LDH) levels (3,4,6-9), glutamate-oxaloacetate transaminase (GOT) levels (3,6,7) and adenosine triphosphatase activity (10,11) during perfusion preservation. However, total adenine nucleotide levels in the tissue have shown definate relationships to function (12), but this test required 250 mg. of tissue.

A number of workers have been interested in the effects of purines in perfusates. Buhl (13) found a precise link between the level of oxypurines in the kidney effluent and warm ischaemia. Infusion of inosine prior to warm ischaemia has prevented the damaging effects observed in the controls (14). The efficient preservation of red blood cells has been enhanced by purines, particularly inosine, adenine and guanosine when added to the pyruvate and phosphate rich preservation medium (15-18).

It was decided to investigate the outcome of adding purines to kidney preservation media. However, before any documentation of results was possible, a reliable method of purine quantification had to be established. This has already been achieved by utilizing an isocratic reverse phase system of H.P.L.C.(19). A modification of this technique, enabling fast isolation of the various purines that exist in the kidney perfusates is described in this paper.

MATERIALS

Animals And Operative Procedure

New Zealand Albino rabbits of 3kg weight were used for all experiments, following the technique of Green (20).

Perfusion Apparatus

The kidneys were perfused at 40mm Hg pressure for 24 hours at 8°C using an extracellular solution which contained 5% dextran 70 as the colloid. This was bubbled with 95% oxygen and 5% carbon dioxide to maintain the pH at 7.4. Various mixtures of purines were added to the perfusate (see Table 1) which contained 5mm pyruvate and 15g/l glucose.

H.P.L.C.

An ALC/GPC 204 High Pressure Liquid Chromatograph consisting of a U6K septumless injector, a dual channel Model 440 ultra-violet (UV) absorption detector and a Model 6000A solvent delivery system was utilized (Waters Associates, Milford, Massachusetts). A 660 solvent programmer and an extra pump (Waters Associates) was also attached to enable gradient elution. The UV detector was linked to a two channel Phillips PM 8252 chart recorder which enabled comparison of the traces at 254 + 280nm simultaneously.

Column

A pre-packed reverse phase μ-Bondapak C_{18} column of 4mm I.D x 30cm (Waters Associates) was employed for all traces at ambient temperature.

Table 1

ADDITIVES TO PERFUSATES

Adenine	1.8mM
Adenosine	5mM
Allopurinol	500mg/l
Guanosine	5mM
Inosine	10mM
IMP	5mM
Ribose	5mM

Eluents

Dipotassium hydrogen phosphate buffer (10mg/l) adjusted to pH 4.5 with phosphoric acid was filtered through a submicron filter prior to use as the low concentration eluent. A mixture of 20% methanol in buffer was used as the solvent for the second pump, the final concentration of methanol programmed to 10%. For isocratic runs the eluent was 4% methanol in buffer.

METHODS

Samples

The samples of rabbit kidney perfusate were diluted six times with 3% perchloric acid to precipitate any protein. After centrifugation and removal of the precipitate they were neutralised by mixing with an equal volume of 60% tri-normal octylamine in freon (1,1,2 - trichlorotrifluoroethane). The neutral topmost layer was stored frozen for analysis after removal by mixing and centrifugation. Each sample was passed through a

submicron filter before injection onto the column. This ensured prolongation of the active life of the column.

Standards

Due to the water insolubility of most purines, the standards were dissolved in IM hydrochloric acid or IM sodium hydroxide before dilution with water and alteration to pH 6.0. These standards, freshly made, were analysed at frequent intervals to check for any column variation, which was not observed in these results.

Conditions

For the isocratic runs a flow rate of 1ml/min was necessary using 4% methanol in buffer with a chart speed of 1cm/5 min. For gradient elution the programmer was set to change the eluent from 0-50% of 20% methanol over a period of ten minutes via the convex curve no. 7, and at a flow rate of 2 ml/min. The chart recorder speed was 1cm/2 min.

Identification

Four methods of identification of components were used. These were retention times, standard addition, absorbance ratios and recycling. Initial classification was achieved by referring to retention times, which were similar in all instances. Known amounts of standard solutions were added and any observed increase in one peak provided further diagnostic evidence. Samples were analysed at 254 and 280 nm consecutively enabling absorbance ratios to be calculated. Provided these ratios agreed with those

of the unknown peaks, then identification was complete. If, however, there was a discrepancy in these ratios, the unidentified peak was recycled through the column six times to check homogeneity. Irrefutable diagnosis of peaks is possible using a combination of all these methods.

RESULTS

The isocratic method of 4% methanol in buffer did not produce good resolution and required a flow of 1ml/min which lengthened the overall elution time, see figure 1. In fact the last peak to be eluted, adenosine, did not appear for over thirty-five minutes after the injection. Certain peaks (allopurinol, inosine, adenine and guanosine) had similar retention times, which were not sufficiently separated by this method. Also, oxipurinol was sometimes completely engulfed by the hypoxanthine peak, thus denying any quantitation of these two compounds.

A technique for delaying the initial peaks, eg. hypoxanthine and oxipurinol, while accelerating the terminal peak, eg. adenosine, was then developed. The gradient elution mode of H.P.L.C. was employed with much success (see figure 2).

The new procedure involved programming 0-10% methanol in buffer over a ten minute period, in such a way that a minimal amount of methanol was present for the first five minutes. This was achieved by using a convex curve (no. 7) and resulted in the appearance of the adenosine peak after 15 minutes. The reversal time was reduced owing to the low final concentration of methanol. This method facilitated the good separation of hypoxanthine and

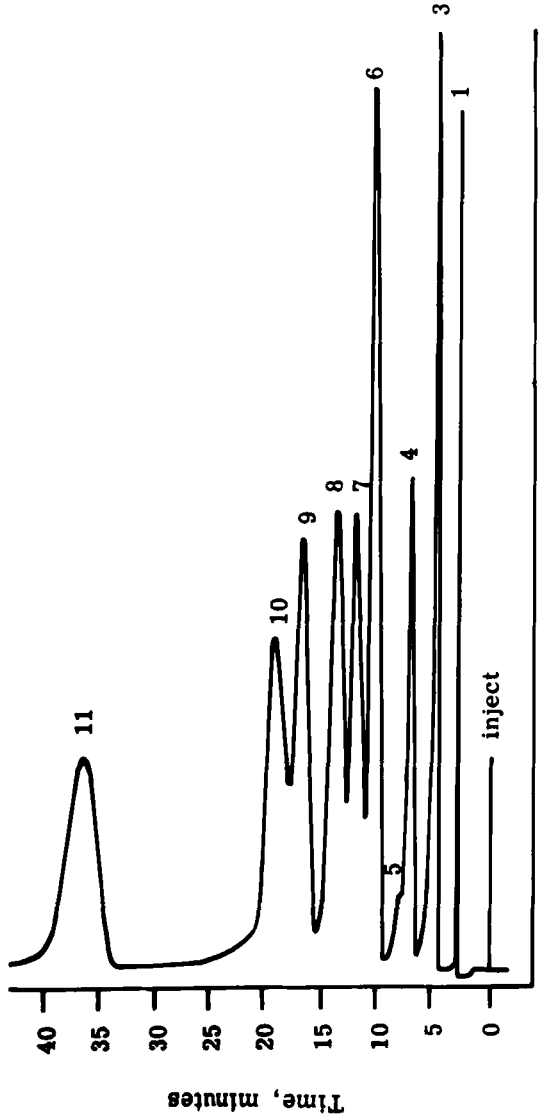

Figure 1. Chromatogram of perfusate absorbance at 254 nm - isocratic method. Conditions: Column, μBondapak C_{18} 4 mm I.D. X 30 cm; pH, 4.5; Flow rate, 1 ml/min; Chart speed, 1 cm/5 min; Detector sensitivity, 0.2 A.U.F.S.; Temperature, ambient; Solvent, 4% CH_3OH in K_2HPO_4 (10 mg/l).

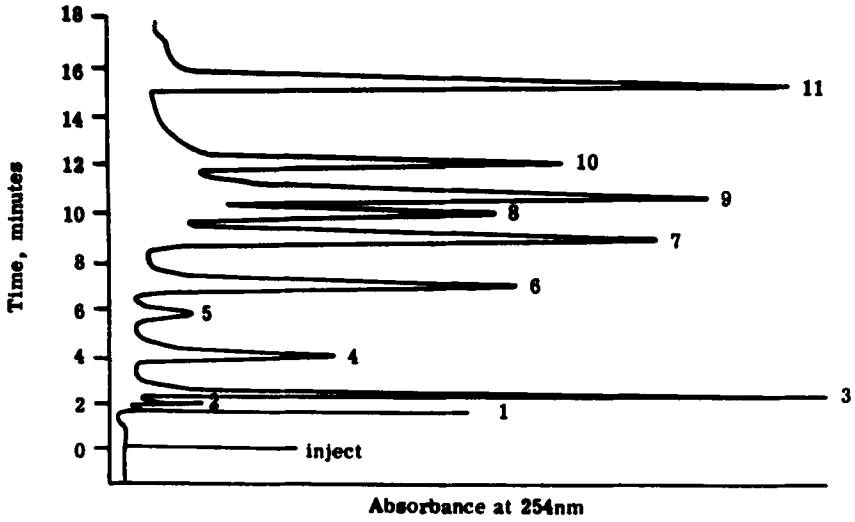

Figure 2. Chromatogram of perfusate absorbance at 254nm - gradient method. Conditions: Column, μBondapak C_{18}4mm I.D. x 30cm; pH, 4.5; Flow rate 2ml/min; Chart speed, 0.5cm/min; Detector sensitivity, 0.2 A.U.F.S.; Temperature, ambient. Solvent: A. K_2HP_4 (10mg/l), B. 20% CH_3OH in K_2HPO_4 (10mg/l) 0% B → 50% B over 10 min via convex curve.

oxipurinol, the adequate isolation of allopurinol, inosine, adenine and guanosine as well as the earlier appearance of adenosine.

DISCUSSION

A previous method of purine analysis (19) has been adapted for use on kidney preservation media. The main improvement is the decrease in retention time that is brought about by inclusion of a gradient elution mode. The speedy completion of a run, less than twenty minutes, will be of great advantage if the purine levels in the kidney perfusion media can be shown to correlate with the viability of the organ, and thus, warrent the use of this method clinically.

Table 2 **INDEX OF PEAK NUMBERS**

Peak number	Compound	Retention time in minutes	Concentration in μg	254/280 absorbance ratio
1	Inosine monophosphate	1.4	1.96	7.21±0.34
	Lactate		0.192	2.36±0.1
	Pyruvate		0.019	4.75±0.13
2	Ribose	1.6	0.075	1.787±0.07
	Uric acid		0.236	0.54±0.02
3	Creatinine	2.2	4.0	∞
4	Hypoxanthine	2.6	0.953	14.06±0.15
5	Oxipurinol	3.3	0.6	5.21±0.06
6	Allopurinol	6.4	2.72	13.03±0.1
7	Inosine	7.2	2.68	7.48±0.07
8	Guanosine	9.4	1.98	2.30±0.02
9	Adenine	10.2	1.35	7.93±0.13
10	5'-deoxy-guanosine	11.6	0.561	2.35±0.04
11	Adenosine	14.8	2.67	6.58±0.05

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REFERENCES

1. Abouna, G.M., Delong, T.G., Pashley, D.H., Sobel, R.E., Ginsburg, J.M. Critical evaluation of viability assays in renal preservation.
Br. J. Surg. 61, 325, 1974.

2. Grundman, R.T., and Pichlmaier, H. Preservation of canine kidneys with hypothermic non pulsatile perfusion.
Arch. Surg. 106, 301, 1973.

3. Liebau, G., Klose, H.S., Fischback, H. and Pilchmaier, H. Simple tests for viability of the hypothermic pulsatile

perfused dog kidney.
Surgery. 70, 459, 1971.

4. Johnson, R.W.G., Anderson, M., Taylor, R.M.R., Swinney, J. Significance of perfusate lactic acidosis in cadaveric renal transplantation.
Br. Med. J. 1, 391, 1973.

5. Baxby, K., Anderson, M., Johnson, R.W.G., Taylor, R.M.R., Swinney, J. Assessment of cadaveric kidneys before transplantation.
Br. J. Surg. 61, 911, 1974.

6. Stephenson, T.P., Hendry, W.F., Ward, J.P., Wickham, J.E.A. Continuous perfusion of human cadaveric kidneys for transplantation.
Br. J. Urol. 47, 37, 1975.

7. Weinerth, J.L., Abbott, W.M. Analysis of injury in complex organ preservation.
Ann. Surg. 180, 840, 1974.

8. Belzer, F.O., Ashby, B.S., Downes, G.L. Lactic acid dehydrogenase as an index of future function of cadaver kidneys during isolated perfusion.
Surg. Forum. 19, 204, 1968.

9. Kohn, M., Ross, H. Lactate dehydrogenase output of the excised kidney as an index of acute ischaemic damage.
Transplantation 11, 461, 1971.

10. Jonsson, O., Lundstam, S., Pettersson, S., and Scherstein, T. Kidney viability: evaluation by determination of Na^+, K^+- ATPase activity and K^+ - transport over cell membranes.
Transpl. Proc. 1X, 1565, 1977.

11. Petterson, S., Class, G., Schenstein T. Correlation between sodium-potassium-stimulated ATPase activity and renal function after transplantation of canine kidneys.
Europ. Surg. Res. 6, 18, 1974.

12. Calman, K.C., Quinn, R.O., Bell, P.R.F., in Organ Preservation. Ed. David E. Pegg , Churchill Livingstone, Edinburgh 1973 ,p 225.

13. Buhl, M.R. Oxypurine excretion during kidney preservation: an indicator of ischaemic damage.
Scand J. Clin. Lab. Invest. 36, 167, 1976.

14. Fernando, A.R., Armitage, D.M.G., Griffiths, J.R., Hendry, W.I., O'Donoghue, E.P.N., Pernett, D., Ward, J.P., Wickham, J.E.A. Enhanced preservation of the ischaemic kidney with inosine.
Lancet, March 13. p555, 1976.

15. Chanutin, A. in modern problems of blood preservation, International Symposium, ed. Seidl , S. and Speilmann, W., Fischer, Stuttgart, 1970, p78.

16. Prins, H.K. and Loos, J.A. in Modern problems of blood preservation, Internatiaon Symposium, ed. Seidl, S. and Spielmann, W., Fischer, Stuttgart, 1970, p110.

17. Warrenderf, E.M. and Rubinstein, D. The elevation of adenosine-triphosphate levels in human erythrocytes.
Blood 42, 637, 1973.

18. Buhl, M.R., Kemp, E. and Kemp, G. Inosine in the preservation of rabbit kidneys for transplantation.
Transpl. Proc. IX, 1603, 1977.

19. Brown, P.R., Krstulovic, A.M., Hartwick, R.A. Analysis of purines and their nucleosides by the reverse phase partition mode of High Pressure Liquid Chromatography.
Adv. Exp. Med. Biol. vol. 76a, 610, 1976.

20. Green, C.J. Rabbit renal autografts as an organ preservation model.
Laboratory Animals, 7, 1, 1973.