*Journal of Chromatography*, 344 (1985) 101—113
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2760

# USE OF A FUNDAMENTAL ELUTION PROTOCOL FOR THE DEVELOPMENT OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ENABLING RAPID SIMULTANEOUS DETERMINATION OF PURINES, PYRIMIDINES AND ALLIED COMPOUNDS COMMONLY FOUND IN HUMAN BIOLOGICAL FLUIDS

GEORGE S. MORRIS* and H. ANNE SIMMONDS

*Purine Laboratory, Clinical Science Laboratories, Guy's Hospital, London Bridge SE1 9RT (U.K.)*

(First received April 1st, 1985; revised manuscript received June 26th, 1985)

## SUMMARY

Many high-performance liquid chromatographic methods for the analysis of nucleosides and bases have been developed for a specific requirement. Most are not directly applicable to the separation of all the purines and pyrimidines found in biological fluids. A method has been developed to separate a wide range of compounds found in human urine and plasma in a single run without unduly long run times. Special attention has been paid to sample preparation. Isocratic systems derived from this method for the analysis of specific compounds and enzyme assay mixtures over even shorter periods are described.

## INTRODUCTION

A large number of procedures have been used for the analysis of nucleosides and bases by high-performance liquid chromatography (HPLC) [1—4]. However, most of the methods described can only accommodate a limited number of compounds in one analytical run, or have inordinately long run times and are not commensurate with a reasonable sample throughput in a diagnostic environment. Rapid isocratic and gradient methods have been reported for the separation of uric acid and other oxypurines [5—7], as have procedures for the measurement of the purine analogues, allopurinol and oxipurinol [8], in addition to the numerous techniques developed for the therapeutically important methylated xanthines [9, 10]. These methods use a variety of primary retention mechanisms, from ion exchange through ion pairing to reversed-phase.

0378-4347/85/$03.30 

Problems are inevitably encountered when trying to evaluate a number of different purines or pyrimidines in biological fluids. This is exacerbated in renal failure and when trying to use the elevated levels of minor purine or pyrimidine bases characteristic of the different inherited metabolic disorders, as a diagnostic tool. Methylated xanthines derived from the ingestion of tea, coffee, chocolate, etc., also confuse the picture, as does the use of antibiotics or other pharmacological agents often essential in the treatment of the condition. The latter are frequently either purine or pyrimidine analogues.

The lack of a suitable system which would allow the simultaneous determination of the large number of purine or pyrimidine nucleosides and their analogues which might be encountered in such situations, in a single run, necessitated the development of a rapid and sensitive method with wider ranging capabilities.

## EXPERIMENTAL

### *Chemicals*

All reference standards (purine and pyrimidines and associated compounds) were of the highest analytical grade available and were obtained through commercial sources (Sigma, Fluka and BDH). Ammonium acetate (analytical-reagent grade) and acetic acid (chromatographic grade) were supplied by BDH. Solvents were obtained from Rathburn Chemicals (Walkerburn, U.K.) and water of sufficient purity for HPLC was produced by double glass distillation of deionised water.

### *HPLC apparatus*

A Waters tri-module automatic system (Waters) equipped with a Model 440 (254/280 nm) dual-wavelength detector and a Model 720 systems controller, or a Model 721 programmable system controller with slightly modified software, was used. Ready to use pre-packed (250 × 4.9 mm) Spherisorb ODS-5 columns (Hichrom), protected by a 2-$\mu$m Rheodyne filter (Anachem) and a Co:Pell ODS (30—38 $\mu$m) resin (Whatman) packed guard column (50 × 5 mm) completed the analytical system. Additional protection was given to the analytical system in the form of a 100-$\mu$m silica-gel-packed (50 × 5 mm) pre-column. The pre-column was added to the analytical system in order to protect the analytical column from the high-aqueous buffers, inevitably used in nucleoside and base work, since problems had been experienced with the rapid degradation of analytical performance caused by the slow dissolution of resin by the buffers. The pumps were protected from particulate matter by the use of in-line filters on the low-pressure side of the system. All filters were regularly cleaned by sonication in 6 *M* nitric acid for 20 min. Pumps and all 316 stainless-steel tubing was regularly "passified" with 6 *M* nitric acid as required. Prior to use all buffers and solvents were vigorously degassed and particulate matter removed by passage through a 0.45-$\mu$m filter under vacuum.

### *Sample preparation*

*Standards.* An approximately 1 m*M* solution of each of the test compounds was made up in 50 ml of glass-distilled water. A 100-$\mu$l aliquot was taken and

added to 3 ml of 0.01 *M* hydrochloric acid. The UV spectrum (190—310 nm) was then recorded on a Perkin Elmer Model 402 spectrophotometer, the exact concentration was then calculated from the extinction coefficient and the spectrum checked for authenticity at pH 2.00 and 10.0. Where the extinction coefficient was unknown, an exact amount of compound was dried in a desiccator to constant weight, weighed and made up carefully before the spectrum was recorded and an extinction coefficient calculated. For characterisation and analysis by HPLC a further 100-$\mu$l aliquot of stock solution was taken and diluted 1:31 with other standards, distilled water, or a mixture of both, prior to injection onto the chromatograph. Stock solutions of stable reference standards were stored at −20°C for future use.

*Plasma.* Fresh heparinised blood collected on ice was centrifuged immediately at 3500 rpm (3000 *g*) for 5 min in a bench centrifuge (Damon Model 7Rx). The plasma was removed and the cellular fraction processed for nucleotide estimations, enzyme assays, whole cell metabolic studies, etc. [11, 12]. A 10% trichloroacetic acid (TCA) solution was then added to an aliquot of plasma while mixing vigorously on a vortex mixer in a 1:1 ratio. The mixture was then spun in a Beckman microfuge at 12 000 *g* for 2 min. The supernatant was back-extracted with water-saturated diethyl ether until the pH of the aqueous phase was greater than 5.00 (approximately four or five extractions). The extract was then either analysed immediately by HPLC or stored at −20°C for later analysis.

*Urine.* 24-h Urine collections (toluene preservative) were gently warmed by immersing the containers in hot water for 0.5 h with occasional shaking to dissolve any precipitate. Aliquots were then taken and stored at −20°C if not processed immediately. Urine was treated in two ways. A 100-$\mu$l aliquot was diluted 1:31 with the HPLC buffer used at the point of injection and 50 $\mu$l were injected directly. Additionally, a further 1-ml aliquot of neat urine adjusted to pH 10.00 was then passed onto a short (ca. 60 × 5 mm) glass column packed with analytical-reagent grade (AG 1-X8, 100—200 mesh) anion-exchange resin in the acetate form (Bio-Rad). The column had previously been washed with 5 ml of glass-distilled water, followed by 1 ml of 0.001 *M* ammonium hydroxide to ensure the correct pH. The column was then sequentially developed with glass-distilled water (5 ml), 0.05 *M* hydrochloric acid (5 ml) and 5 ml of a solution containing 30 g/l sodium chloride in 0.02 *M* hydrochloric acid. The eluent from each of the above washings was collected separately and 50-$\mu$l samples of each were injected onto the chromatograph. For concentrated urines (i.e. those of more than 500 mOs/kg) neat urine was diluted before use to give an osmolality of around 200 mOs/kg.

Adoption of this lengthy procedure for urines proved necessary because of the very large number of UV-absorbing components commonly found (more than 100 [13]). The dual approach using a direct dilution of urine, in addition to the fractionated samples, was essential for two reasons. First, as a check on the effectiveness and efficiency of the fractionation process; second, not all the compounds of interest eluted from the preparative anion column and consequently could only be detected in whole urine.

*Experimental procedure for the development of HPLC protocol*

Initially, the pH, type and the amount of salt to be added to the water base solvent was determined. Ammonium acetate appeared to give the most promising results. A 100 m*M* solution was diluted in nine uniform steps to 10 m*M* and sample chromatograms were run at each step over a range of pH values (2.00—6.00). From this procedure the optimum buffer concentration and pH were calculated, i.e. the composition and pH that maintained the most symmetrical peak shape of all the test compounds, together with a large enough retention range and absolute retention to allow for the subsequent development of resolution. In practice, this meant that the capacity factors ($k'$) lay in the range of approx. 5—15. During this process the need for gradient elution became apparent.

Due to widely varying retention times — giving rise to extraordinarily long elution times — it was not possible to work on all compounds of interest at one time. Consequently, the various classes of compounds had to be treated separately during the rest of the development phase. Four groups were selected: (1) the pyrimidine bases and nucleosides; (2) the oxypurines and pyrazolopyrimidine analogues; (3) the guanosine and inosine series and their corresponding deoxynucleosides; (4) the adenine-based series and the more common methylated xanthines. These groupings were drawn up on the following premises. Firstly, chemical analogy; secondly, published HPLC elution behaviour; thirdly, and possibly the most important, compounds that required rapid simultaneous assay. Subsequent steps were performed using each group separately and individual isocratic procedures developed for each, the different procedures being combined at a final stage in order to produce a continuous system.

A gradient of the chosen basal buffer (40 m*M* ammonium acetate, pH 5.00, the same for all the groups) was run against methanol (0—100%) for each of the compounds in the group and for a mixture of all the compounds in the group. From this the percentage of methanol at which the compound with the greatest $k'$ value eluted was noted. Each of the compounds and then the mixture were run in an isocratic mode with the mixture of buffer and methanol at the percentage essential to elute the compound with the largest $k'$ value. After this the percentage of methanol was adjusted so that the $k'$ value of the most strongly retained compound lay between 5 and 10, if necessary. In three of the groups this proved unnecessary. Next, the isocratic portion of the experiment was repeated with acetonitrile in place of methanol, the percentage being adjusted in relation to the basal buffer to produce a $k'$ value for the last eluting compound roughly comparable to that produced with methanol (see Table I). The procedure was then repeated with tetrahydrofuran.

After the percentage composition of all three organic modifiers had been determined, the isocratic experiments continued, using the same procedure, but with an eluent consisting of methanol-modified buffer—acetonitrile buffer (50:50), followed by a series of elutions using methanol-modified buffer—tetrahydrofuran-modified buffer (50:50), then with a mixture of acetonitrile-modified buffer—tetrahydrofuran-buffer (50:50). Finally, in this series of isocratic elutions, a mixture of each of the three buffers (1:1:1) was made up and the separate compounds of the group and the mixture run once more. On com-

pletion, chromatograms from the group were considered together and an optimised quaternary mobile phase postulated from the retention data and information given by the above isocratic experiments. The proposed eluent was then prepared and the isocratic procedure repeated. Minor adjustments were made in light of this final isocratic stage in the development sequence. When the

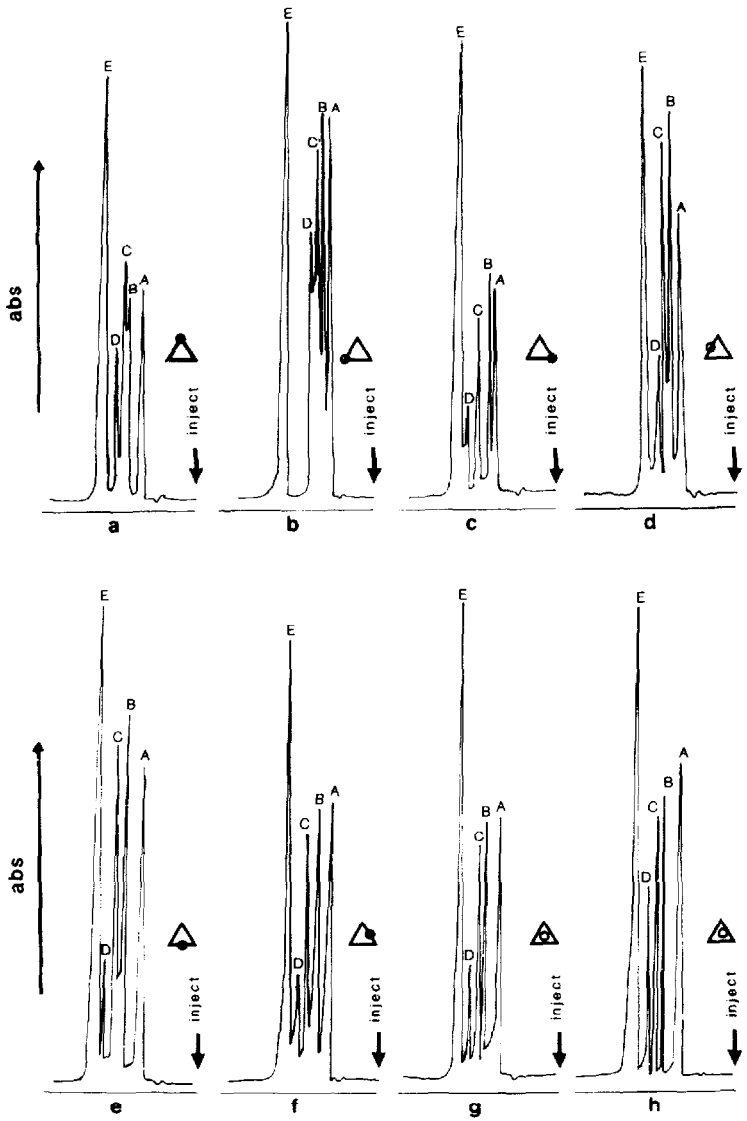


Fig. 1. Chromatograms of the series of seven isocratic experiments performed in order to develop the resolution of the oxypurines (group 2) (a—g), and the fully optimised separation derived from the experiments (h). The triangle to the right of each chromatogram represents the selctivity triangle, with the top point designated methanol, the bottom left acetonitrile and right tetrahydofuran. The circle on each triangle expresses the nature of the organic modifier used in each experiment. The chromatograms were recorded at 254 nm. Peaks: A = uric acid; B = hypoxanthine; C = xanthine; D = oxipurinol; E = allopurinol.

separations for each group had been completed, the eluents developed for each were connected into a single continuous gradient system using information derived from the isocratic studies and the initial gradient stages of the development phase.

*Development of derived isocratic systems*

In an endeavour to increase sample throughput and to cut solvent consumption, the possibility of using the fully developed system as the basis of different isocratic systems for assays of specific purine or pyrimidine compounds without the need for a full developmental sequence was investigated. The isocratic systems were produced by simply running a mixture of the compounds to be analysed on the gradient system. After checking for adequate resolution, a spike of the mixture was added to whatever matrix would be present in the samples to be analysed. The sample was then re-run and the point on the gradient at which the last compound/peak eluted recorded and the resolution of the peaks of interest re-checked. The mix was then run isocratically with the composition of buffer corresponding to the point on the gradient at which the last peak eluted. If, as was normally the case, the separation was still adequate, the newly developed system was set up on another instrument and calibrated prior to use. Where the separation proved inadequate, a marginal reduction in the organic concentrations of the buffer, below the predicted values, was all that was normally required in order to maintain chromatographic separation.

## RESULTS

Representative chromatograms from the development sequence of the oxy-

TABLE I

RESULTS FROM THE SERIES OF EXPERIMENTS INVOLVED IN THE DEVELOPMENT SEQUENCE OF THE "GROUP 2" ISOCRATIC SYSTEM

The results show how the various compounds behave under the various eluent conditions

| Eluent composition* | Compound retention $k'$ value | | | | |
|---|---|---|---|---|---|
| | Uric acid | Hypoxanthine | Xanthine | Oxipurinol | Allopurinol |
| Acetate | 1.77 | 3.20 | 3.56 | 4.40 | 5.89 |
| 0—100% Methanol | 1.25 | 1.30 | 1.41 | 1.90 | 2.03 |
| A: Methanol 13.0% | 0.55 | 1.00 | 1.05 | 1.33 | 1.66 |
| B: Acetonitrile 8.0% | 0.28 | 0.33 | 0.64 | 0.72 | 1.55 |
| C: Tetrahydrofuran 3.0% | 0.77 | 0.88 | 1.22 | 1.55 | 1.77 |
| D: A—B (50:50) | 0.50 | 0.81 | 0.91 | 1.11 | 1.61 |
| E: B—C (50:50) | 0.44 | 0.78 | 1.00 | 1.33 | 1.66 |
| F: A—C (50:50) | 0.66 | 1.16 | 1.73 | 1.66 | 1.90 |
| G: A—B—C (1:1:1) | 0.67 | 1.06 | 1.22 | 1.44 | 1.77 |
| H: Idealized eluent (see Table II for composition) | 0.61 | 1.00 | 1.22 | 1.44 | 1.85 |

*All eluents were made up with 40 m*M* ammonium acetate pH 5.00.

purines and pyrazolopyrimidines separation (group 2) are shown in Fig. 1. The results from this series of experiments are fully tabulated in Table I, and demonstrate the marked degree of variation in retention and resolution of the various compounds under different eluent compositions of roughly comparable eluting power (solvent strength) depending on the degree of $H^+$ donation or acceptance and dipole moment of the specific eluent system. Similar data (not shown) were obtained for each of the other groups. Table II shows the optimised eluent composition for each of the developmental groups, described in Experimental, in percentage volume terms. Fig. 2 illustrates a chromatogram of reference standards run on the fully developed gradient system, demonstrating the resolution and even separation of the compounds of interest. The chromatographic conditions of the fully developed system are shown in Table III.

TABLE II

OPTIMAL BUFFER COMPOSITIONS FOR THE FOUR GROUPS OF COMPOUNDS DISCUSSED IN TEXT

| Group No. | Percentage composition total eluent (v/v) | | | |
|---|---|---|---|---|
| | Acetate | Methanol | Acetonitrile | Tetrahydrofuran |
| Group 1 | 94.00 | 4.80 | 1.00 | 0.20 |
| Group 2 | 84.22 | 12.17 | 2.05 | 1.56 |
| Group 3 | 83.01 | 12.13 | 2.29 | 2.57 |
| Group 4 | 80.50 | 11.31 | 7.61 | 0.58 |

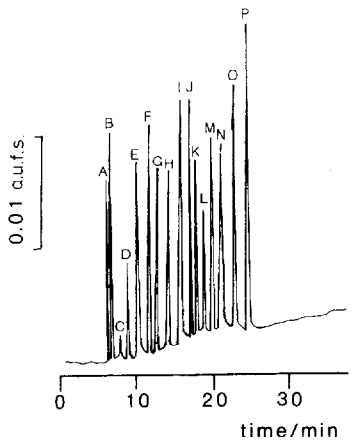


Fig. 2. A mixed standard run at 254 nm using the ultimately derived gradient system, as described in Experimental and Table III, illustrating the separation obtained for a selection of the compounds frequently encountered during studies of human purine metabolism. Other compounds readily resolved under these conditions are listed in Table IV. Peaks: A = pseudouridine; B = uracil; C = tyrosine; D = uric acid; E = uridine; F = hypoxanthine; G = xanthine; H = oxipurinol; I = allopurinol; J = inosine; K = guanosine; L = deoxyinosine; M = deoxyguanosine; N = adenine; O = adenosine; P = deoxyadenosine.

TABLE III

OPERATING DETAILS OF THE FULLY DEVELOPED GRADIENT SYSTEM FOR THE ANALYSIS OF NUCLEOSIDES, BASES AND RELATED COMPOUNDS

| | |
|---|---|
| Column | A 250 × 5.9 mm column packed with Spherisorb ODS-5 5-$\mu$m reversed-phase $C_{18}$ resin |
| Eluent composition | (A) 40 m*M* Ammonium acetate adjusted with glacial acetic acid to pH 5.00, and 1% methanol (v/v) added after pH adjustment.<br>(B) Methanol—acetonitrile—tetrahydrofuran (80:10:10). |
| Volume flow-rate | Constant, 1 ml/min |
| Gradient | 0—30% Eluent B linearly over 30 min. |
| Equilibration period | Eluent A 12 ± 2 min. |
| Column temperature | Ideally 25°C, but room temperature is adequate provided it is below 30°C |
| Detector | Dual 254/280 nm UV detector as a minimum; on-line radiodetection has also successfully been used [19]. |

TABLE IV

TYPICAL RETENTION DATA AND 280/254 nm RATIOS OF 26 PURINES, PYRIMIDINES AND ASSOCIATED COMPOUNDS FREQUENTLY ENCOUNTERED DURING THE ANALYSIS OF HUMAN BODY FLUIDS

Compounds are shown in elution order. The data in this table only refer to a specific column on a particular day with a given set of eluents, therefore variation can be expected.

| Compound | Retention time (min) | 280/254 nm Ratio |
|---|---|---|
| Orotidine | 3.57 | 0.73 |
| Orotic acid | 4.19 | 1.90 |
| Pseudouridine | 5.73 | 0.36 |
| Uracil | 6.31 | 0.16 |
| Tyrosine | 7.62 | 2.30 |
| Uric acid | 8.39 | 3.27 |
| Uridine | 9.56 | 0.26 |
| Hypoxanthine | 10.87 | 0.06 |
| Xanthine | 12.61 | 0.63 |
| Guanine | 13.23 | 0.61 |
| Oxipurinol | 14.17 | 0.17 |
| Allopurinol | 16.73 | 0.08 |
| Oxipurinol riboside | 17.72 | 0.19 |
| Inosine | 18.00 | 0.06 |
| Allopurinol riboside | 18.53 | 0.11 |
| Guanosine | 18.72 | 0.60 |
| Deoxyinosine | 19.12 | 0.06 |
| Deoxyguanosine | 19.91 | 0.62 |
| 7-Methylguanine | 20.20 | 0.57 |
| 8-Hydroxy-7-methylguanine | 20.50 | 0.60 |
| Adenine | 21.46 | 0.14 |
| Tryptophan | 22.11 | 1.83 |
| Adenosine | 23.69 | 0.16 |
| Deoxyadenosine | 26.15 | 0.16 |
| Theophylline | 27.76 | 1.25 |
| Caffeine | 29.32 | 1.54 |

A list of the typical retention times and 280/254 nm ratios of 26 compounds regularly found as constituents of human body fluids is shown in Table IV. Approximately 90 to 100 different compounds have been analysed on this system and their chromatographic data and 254/280 nm ratios catalogued. The importance of the latter is illustrated below.

Fig. 3 shows typical chromatograms derived from the plasma and urine of a normal control subject on a low-purine caffeine-free diet and illustrates the

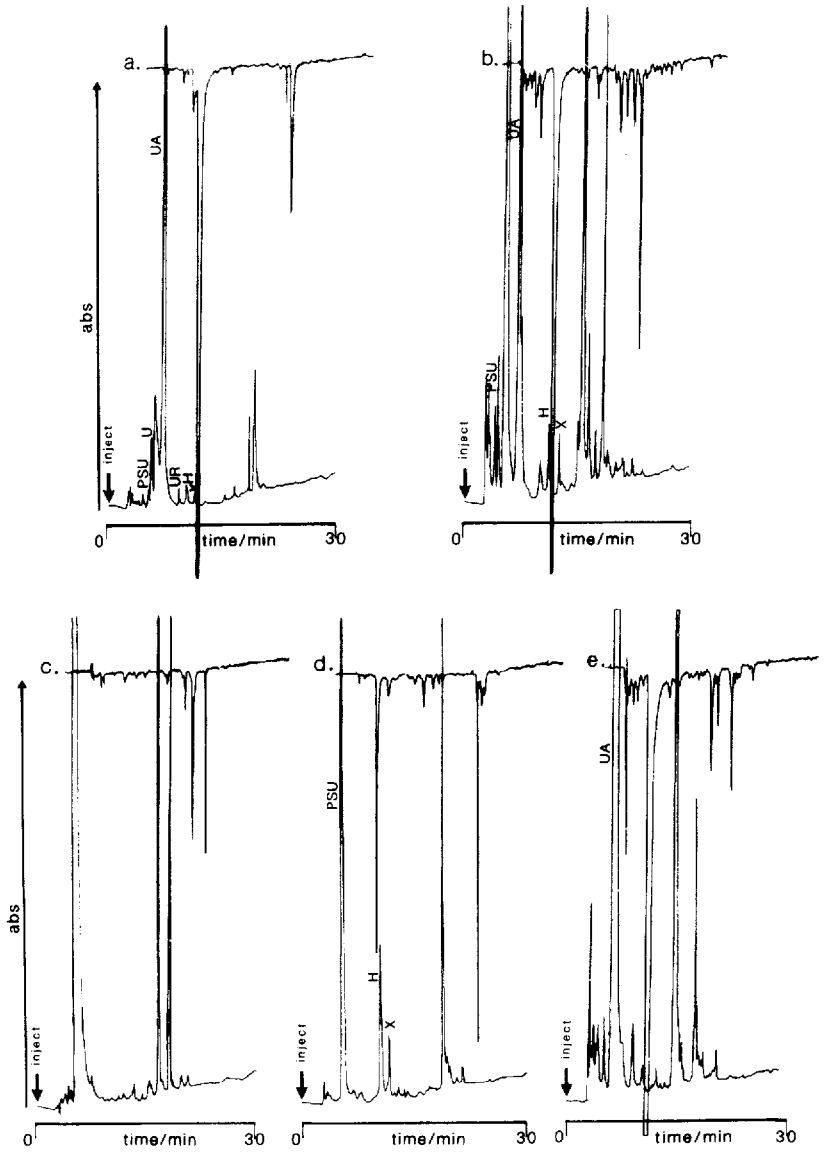


Fig. 3. Chromatograms of the plasma (a) and urine diluted 1:31 (b), together with urine fractions 1, 2 and 3 (c, d and e, respectively), of a subject on a low-purine caffeine-free diet. The lower trace of each chromatogram was recorded at 254 nm UV, the upper at 280 nm; both at 0.05 absorbance units full scale (a.u.f.s.). PSU = Pseudouridine; U = uracil; UA = uric acid; UR = uridine; H = hypoxanthine; X = xanthine.

TABLE V

RESULTS OF EXPERIMENTS SHOWING YIELDS OBTAINED IN THE DIFFERENT ANALYTICAL PROCEDURES

| Compound | Recovery (%) | | | |
|---|---|---|---|---|
| | Standard after TCA extraction | Standard through fraction column | Spiked plasma after TCA extraction | Spiked urine through fraction column |
| Orotidine | 95 | 87 | 88 | 86 |
| Orotic acid | 102 | 88 | 94 | 86 |
| Pseudouridine | 92 | 90 | 87 | 90 |
| Uracil | 90 | 89 | 85 | 94 |
| Uric acid | 100 | 103 | 98 | 98 |
| Uridine | 94 | 89 | 82 | 89 |
| Hypoxanthine | 93 | 92 | 80 | 81 |
| Xanthine | 94 | 91 | 91 | 88 |
| Oxipurinol | 96 | 100 | 93 | 95 |
| Allopurinol | 98 | 87 | 84 | 83 |
| Adenine | 104 | 99 | 101 | 91 |
| Adenosine | 97 | 86 | 82 | 80 |
| Deoxyadenosine | 96 | 89 | 80 | 77 |
| Mean | 96.2 | 91.5 | 88.1 | 87.5 |

need to fractionate urine specimens before HPLC analysis, in order to achieve meaningful results qualitatively and quantitatively.

Table IV demonstrates that some of these (caffeine, theophylline) have retention times similar to the adenine-based compounds but very different 280/254 nm ratios. This underlines the importance of having at least two-channel detection for the calculation of detector response ratios as an additional guide to compound identification. Table V indicates the excellent yields obtained for a variety of components commonly encountered in biological fluids in the different procedures described in Experimental.

From Table IV it may be seen that a specifically derived isocratic system could be readily devised using the method described for the more rapid throughput of a large number of samples. An example is the assay of different purine/pyrimidine enzymes when all that is required is the separation of a very limited number of components, i.e. substrate/product (such as inosine/hypoxanthine in the assay of the enzyme purine nucleoside phosphorylase [7]).

## DISCUSSION

The separation described in this paper was developed using a basic elution protocol similar to that proposed by Glajch et al. [14] and Snyder [15] for generalised isocratic reversed-phase method development. This approach lays down a precise and defined experimental procedure by which any possible

separation can be developed. It is fundamentally based on the solvent selectivity triangle originally described by Rohrschneider [16], and developed by Snyder [17, 18] which allows for the "fine tuning" of selectivities where a solvent from any given selectivity group will not produce the desired effect alone. In any reversed-phase system the three solvents that are best able to influence selectivity and are compatible in terms of miscibility etc., are methanol, acetonitrile and tetrahydrofuran. Water is added as a strength-adjusting agent to provide for a proper retention range. In practice, various salts have to be added to the base solvent, water, in order to maintain the chromatographic peak shape of the compounds of interest.

The wide range of retention values coupled with the large number of UV-absorbing compounds found in biological fluids frequently necessitates the use of gradient elution. Therefore, modification to the basic methodology of Glajch et al. [14] and Snyder [15] was required. Development of the gradient elution protocol should have proved difficult using a binary pumping system for what is essentially a quaternary system. However, group 1, the pyrimidines — most of which had low $k'$ values and consequently eluted early — only required a low concentration of methanol and minimal amounts of acetonitrile in order to produce reliable and reproducible separations. Thus, it proved possible to incorporate 1% methanol with the acetate base buffer. A mixture of 40 m*M* acetate (pH 5.00)—1% methanol (1:1) was therefore used at the start of the gradient, i.e. buffer A.

Of all four groups, group 2 (the oxypurines and the pyrazolopyrimidines), required the most rigorously controlled eluent composition in order to produce robust separations over the long term within the constraint of reasonable elution times. To some extent all the other groups had to fit around them. The optimal composition of the eluent for their separation was found to be ammonium acetate (pH 5.00)—organic modifier (84.2:15.8), made up as follows: methanol—acetonitrile—tetrahydrofuran (77:13:10). However, no practical loss of resolution was noted if the buffer's organic modifiers were adjusted to methanol—acetonitrile—tetrahydrofuran (80:10:10). This proved very useful for the dovetailing in of the guanine and inosine series, where the separation appeared to be sensitive to the relative proportion of acetonitrile to tetrahydrofuran. The ideal concentrations determined for isocratic elution were not very different from the adjusted organic modifier concentrations which accommodated the oxypurines and pyrazolopyrimidines already mentioned.

The final group, the adenine series and the methylated xanthines, relied on an exquisitely sensitive balance of methanol and acetonitrile concentration, with only a very small amount of tetrahydrofuran. However, the separation of these compounds was not particularly stable and was column-dependent. This, together with the very different composition of the organic modifiers required for these compounds in comparison with the other groups, gave rise to a problem which has not been satisfactorily resolved in its entirety. At least, in the interim, the solution has been to rely on the relatively large and sufficiently varied $k'$ values of these compounds to produce a reasonably stable and reproducible separation at the end of the chromatogram. The use of a gradient elution profile (an increasing solvent strength with time) effected a form of non-idealized separation, but at the expense of a longer run time and with less

than optimal resolution. Further work is obviously required to resolve this problem, which is in part due to the differing chemical nature of the two groups of compounds placed together in an artificial assemblage during the development of the separation. The long-term solution will probably involve the use of a third pump and eluent.

The methylated xanthines, being a rather hydrophobic group of compounds, are strongly retained by the column primarily on the basis of their solubility in the stationary phase. Any slight change in the composition of the organic proportions of the stationary phase in relation to the mobile phase is thus likely to have an exaggerated effect on their chromatography in comparison with the others analysed by the system, which are in general more polar. This was particularly true with respect to the adenine series which are considerably more basic than most of the other purines and pyrimidines and were consequently retained longer by the column than would reasonably be expected, by reason of a reversed-phase—ion-exchange mixed-mode retention mechanism. Given the pH of the buffers and the lowish carbon loading of the column packing material (even though it is fully end-capped) ionic interactions are likely to predominate in the retention mechanisms of the adenine compounds. This theory is borne out to some extent by the very similar retention times seen on this system for a large range of the adenine compounds, despite considerable variety in side groups which should, in theory, give rise to greater variation in $k'$ values on a reversed-phase system than they do in fact exhibit. This concept is supported by the anomalous chromatographic behaviour observed for 1-methyladenosine. The retention time for 1-methyladenosine was much shorter than the other adenine-based compounds which was the reverse of what was expected from the behaviour of other methylated purines relative to their parent compounds and it exhibited a retention much nearer to that expected for the adenine series, purely on the basis of non-ionic considerations. Therefore, any change in ionic strength or pH will readily change the retention pattern of the group within themselves and in relation to the methylated xanthines.

Thus, when the methylated xanthines and the adenine series were analysed together the conflicting factors affecting the retention and resolution of each became critical. The correct set of parameters for one did not allow a proper separation of the other. Compromise was, therefore, essential. Consequently, all these compounds are chromatographed under less than ideal conditions and the slight, normal, day-to-day variation in operating conditions becomes more critical in this portion of the chromatogram than elsewhere.

Mixed-mode retention may also play a part in other sections of the chromatogram, but not in such a practically obvious manner. The ionic/ion suppression effects are probably predominant in most of the system and are important in the maintenance of peak shape. This would probably be true for all "reversed-phase" separations of compounds that exhibit any degree of polarity.

It is certainly a factor on the occasions when the derived isocratic systems required a lower than predicted organic concentration, or slight change in pH/ion concentration, in order to maintain peak shape and resolution. Such a situation would normally only arise when the compounds of interest were closely grouped with one having a very different retention time from the rest, when such slight empirical adjustments might be required.

The present system has been devised from a knowledge of the essential fundamental theoretical work-up necessary to achieve satisfactory chromatographic separations. It is based around a number of different separation protocols and underlines the success of such an approach even when dealing with complex biological fluids. The system has proved capable of separating a very large number of different purines, pyrimidines and their analogues, in a single run over a much shorter period of time than has proved possible hitherto. It has proved reliable and reproducible throughout the life of many columns, without the need for change in operating conditions. The flexibility of the system is further underlined by its adaptability to the solution of specific problems if and when they should occur as, for example, the rapid assay of different purine enzymes [7] using a derived isocratic method to replace the full gradient system.

ACKNOWLEDGEMENT

These studies were supported by a grant from the Medical Research Council.

REFERENCES

1 R. Eksteen, J.C. Kraak and P. Linssen, J. Chromatogr., 148 (1978) 413.
2 W. Voelter, K. Zech, P. Arnold and G. Ludwig, J. Chromatogr., 199 (1980) 345.
3 P.R. Brown, Cancer Invest., 1 (1983) 527.
4 M.J. Bennett and K.H. Carpenter, Ann. Clin. Biochem., 21 (1984) 131.
5 O.C. Ingebretsen, J. Borgen and M. Farstad, Clin. Chem., 28 (1982) 496.
6 R. Boulieu, C. Bory, P. Baltassat and C. Gonnet, J. Chromatogr., 233 (1982) 131.
7 L.D. Fairbanks, A. Goday, G.S. Morris, M.F.J. Brolsma, H.A. Simmonds and T. Gibson, J. Chromatogr., 276 (1983) 427.
8 R. Boulieu, C. Bory, P. Baltassat and C. Gonnet, J. Chromatogr., 307 (1984) 469.
9 J.L. Blauch and S.M. Tarka, Jr., J. Food Sci., 48 (1983) 745.
10 M. Wenk, B. Eggs and F. Follath, J. Chromatogr., 276 (1983) 341.
11 A. Goday, H.A. Simmonds and G.S. Morris, Clin. Exp. Immunol., 56 (1984) 39.
12 L.D. Fairbanks, H.A. Simmonds, G.S. Morris, E. Carapella de Luca, R.J. Levinsky, C.H. Rodeck and R.L.K. Chapman, in Third International Symposium on Inborn Errors of Metabolism in Humans, Munich, 1984, Karger, Munich, 1984, p. 43.
13 I. Molnár and C. Horváth, J. Chromatogr., 143 (1977) 391.
14 J.L. Glajch, J.J. Kirkland, K.M. Squire and J.M. Minor, J. Chromatogr., 199 (1980) 57.
15 L.R. Snyder, J.L. Glajch and J.J. Kirkland, J. Chromatogr., 218 (1981) 299.
16 L. Rohrschneider, Anal. Chem., 45 (1973) 1241.
17 L.R. Snyder, J. Chromatogr., 92 (1974) 223.
18 L.R. Snyder, J. Chromatogr., Sci., 16 (1978) 223.
19 H.A. Simmonds, A. Goday, G.S. Morris and M.F.J. Brolsma, Biochem. Pharm., 33 (1984) 763.