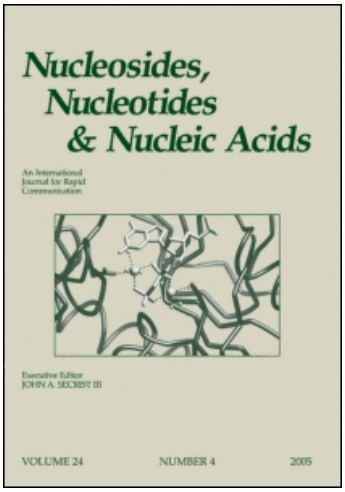

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Development of a Purification Procedure for the Isolation of Nucleosides from Urine Prior to Mass Spectrometric Analysis

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DEVELOPMENT OF A PURIFICATION PROCEDURE FOR THE ISOLATION OF NUCLEOSIDES FROM URINE PRIOR TO MASS SPECTROMETRIC ANALYSIS.

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ABSTRACT : A chromatographic separation of nucleosides from urine has been developed in order to facilitate their mass spectrometric analysis for clinical diagnosis. A number of chromatographic resins were studied in order to develop an effective and efficient purification procedure. The optimized sequential protocol comprises a centrifugation, acidification and neutralization step, followed by application of an affinity chromatographic column and finally further separation on an acidic cation exchange column and a basic anion exchanger. This scheme shows effective clean-up of a standard radiolabelled nucleoside with a recovery of 92.5%, and recovery of nucleosides added to urine samples before extraction showed recoveries of 72 - 82%.

A number of studies have indicated that the levels of modified nucleosides excreted in the urine, believed to originate primarily from tRNA, are potential clinical markers for cancer diagnosis[1]. Such modified nucleosides can be effectively characterised by mass spectrometric analysis[1,2]. The levels excreted are increased in the presence of a tumor when expressed relative to creatinine excretion and have been shown to correlate with the stage of the malignancy[3], decrease rapidly with effective treatment of the disease[4] and increase before any recurrent disease is detected, allowing the monitoring of a patients progress after treatment[5]. However prior to the effective detection and quantitation of these molecules in the urine, a purification procedure is required in order to resolve the nucleosides from other components of the urine, a complex mixture of many compounds in concentrations far greater than those of the modified nucleosides, these contaminants potentially obfuscating nucleosides in their assays[6].

Several previous studies used a variety of different procedures to purify the modified nucleosides from urine, but they did not then employ mass spectrometric analysis of the nucleoside fraction[7], MS analysis being a process in which the cleaner the sample the greater the ease, sensitivity and accuracy of detection, identification and quantitation. In this report a number of chromatographic resins, anion and cation exchangers, and an affinity chromatography resin, were tested in order to determine their effectiveness in separating the nucleosides from other, generally more abundant, "contaminants" found in the urine, in the hope of achieving the separation of a fraction that contains the nucleosides and as few of the other urinary components as possible. These resins are described below.

Phenylboronate affinity resins (derivatized polyacrylamide gels) have been widely used in past purification procedures of nucleosides because of their ability to selectively bind compounds, such as nucleosides, which contain cis-diol groups[7], at pH levels above 7.5 and elute these compounds below pH 6.5[8]. Chelex-100 has also been used to separate nucleic acid derivatives[9]; this resin binds metals and therefore is equilibriated in a source of Cu^{2+} ions followed by an ammonium solution, forming a resin-copper-ammonium complex which acts as a basic anion exchanger.

Dowex ion exchange resins, produced by reacting a cross-linked polystrene matrix with specific functional groups, have also been utilized for biological separations of nucleosides and nucleotides[10] and a number of metal ions in geological samples[11]. Two types of Dowex resin (Dowex-1 and Dowex-50W) were investigated; Dowex-1 (a strong anion exchanger) is produced by reacting the matrix with chloroether and then reacting the chloro groups with tertiary amines to produce an exchanger that is ionised at all but very alkaline pHs. Dowex-50 (a strong cation exchanger) is produced by the sulphonation of the matrix and is ionised at all but very low pHs.

Two types of Sephadex resins (QAE-Sephadex and SP-Sephadex) have been used sequentially to fractionate different classes of compounds from plant extracts[12], they have also been used individually, for example to separate thiamin and thiamin phosphate esters (SP-Sephadex)[13] and to separate natural ribonucleotides (QAE-Sephadex)[14]. These two ion exchangers activities were therefore also considered of potential value for the separation of the nucleoside fraction from other urinary components. Sephadex resins are bead-formed media based on cross-linked dextran[15], QAE (quarternary aminoethyl) Sephadex is a strongly basic anion exchanger which has diethyl-(2-hydroxy-propyl) aminoethyl groups attached by ester linkage to the dextran chains. SP Sephadex is a strongly acidic cation exchanger whose functional group is a sulphopropyl group.

Results and Discussion

In respect of acquisition, handling and nucleoside estimation, a 5ml urine sample was considered ideal : the expected main components of a 5ml sample of urine[6] are listed in Table 1. These

Table 1. Major constituents of urine.

Urinary component	Concentration (mg, unless otherwise stated) per 5ml urine
Urea	14.7
Amino acids	0.84
Creatinine	5
Sugars	1.25
Catecholamines	0.5μg
Nucleic acid bases	0.043
Uric acid	1.6
Ascorbic acid	0.183
Inositol	0.48
Modified nucleosides	0.023

components were examined in a series of chromatographic separations in order to develop a protocol to purify the nucleoside fraction to the maximal extent.

An Affi-Gel 601 column was prepared and used as described in the experimental section. As shown in figure 1, the non-cis-diol compound, uracil, was eluted from the phenylboronate in the ammonium acetate fraction whilst the cis-diol containing compound, inosine, was eluted in the formic acid fraction. In addition inorganic salts are also eluted in the ammonium acetate fraction, hence the nucleoside fraction is effectively desalted. Thus with this type of resin, the contaminants of the nucleoside (formic acid) fraction would be expected to be sugars containing cis-diols (the major urinary sugar being ribose), catecholamines, non-cyclic nucleotides, inositol and ascorbic acid[6]. These contaminants, with the exception of the catecholamines and nucleotides, occur in the urine in concentrations higher than the modified nucleosides (see Table 1) and so complicate any mass spectra produced from the analysis of the fractions. Therefore other resins activity were considered in order to assess their potential for the removal of these contaminants. As the major difference between the nucleosides and most of the other cis-diol-containing components of the urine is the nucleosides basic property, ion-exchange resins theoretically capable of exploiting this difference were investigated.

A Chelex-100 column was set up and eluted as in the experimental section, with ribose acting as a standard of a cis-diol "non-basic" compound (the elution profile of ribose would be expected to mimic other non-basic cis-diols such as inositol and ascorbic acid). Given that the less basic the

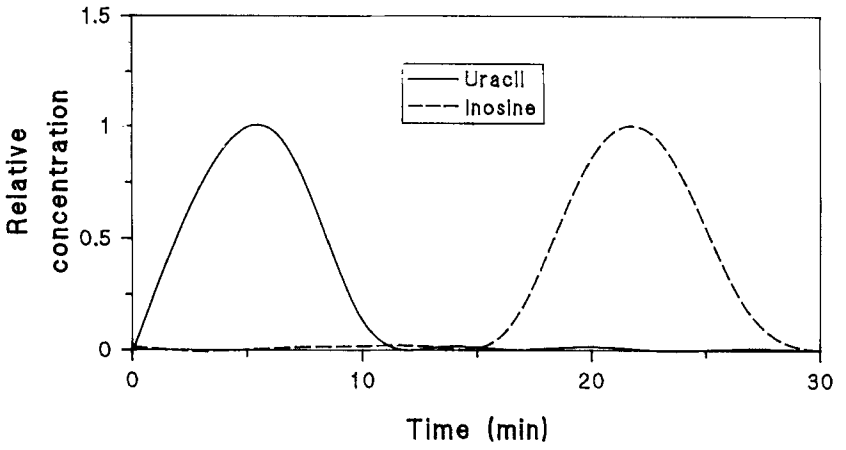

Figure 1. Affi-Gel 601 chromatography of urinary components. Elution of inosine (cis-diol) and uracil (non-cis-diol) are continuously monitored by absorbance at 254nm. For further details see text.

nucleoside the less likely it is that it will be bound and will therefore be eluted in the same way as non-basic compounds, a weakly basic nucleoside (xanthosine, as it had been shown in preliminary investigations to be the first nucleoside eluted) and ribose were used in order to monitor the separation. Xanthosine and ribose are eluted in the same fraction from the Chelex-100 column (Fig 2) and so although this column can separate the more basic nucleosides such as cytidine or adenosine from sugars, as previously also reported by Goldstein[9], these more basic nucleosides require elution with 1M NH_4OH, and it will not separate the weakly basic nucleosides and the non-basic urinary components.

The Dowex-1 column (Fig. 3) was unable to separate the nucleosides from the ribose. The more-basic nucleosides (*e.g.* cytidine) are eluted rapidly whereas the less-basic nucleosides (*e.g.* inosine) and sugars are eluted later, but sugars are seen to contaminate both nucleoside fractions. The Dowex-50W column (Fig 4) allows the separation of sugars from many nucleosides of varying basicity (xanthosine, less-basic and cytidine, more-basic), and the same separation of ascorbic acid as with ribose from these compounds is seen (as expected due to the non-basic properties of ascorbic acid). The sugar fraction is eluted by 5ml water whilst the nucleoside fraction is eluted by the 50ml ammonium formate (adjusted to pH 4.86). Uridine however is shown to be eluted in the sugar fraction and so exhibits a different elution profile than that seen with Chelex-100 resin (in which xanthosine was eluted before uridine[9]), despite both resins relying upon the nucleosides basicity for their separation. It is therefore

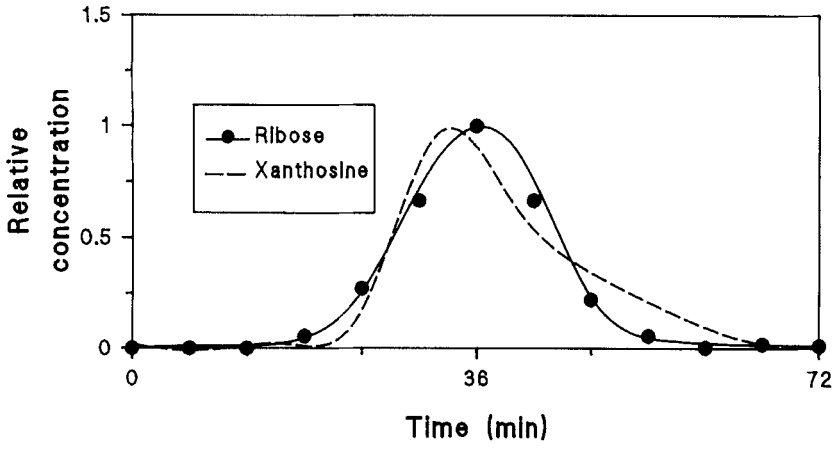

Figure 2. Chelex-100 chromatography of urinary components. Elution of xanthosine is monitored by absorbance at 254 nm, of ribose by phenyl sulphuric assay. For further details see text.

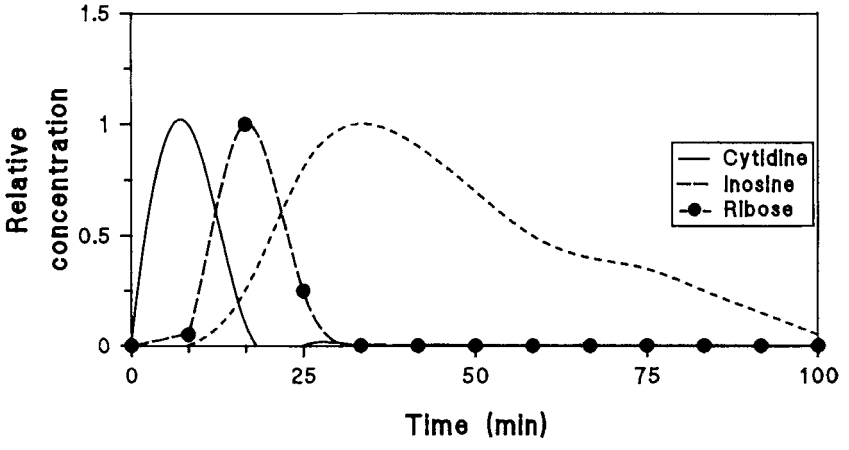

Figure 3. Dowex-1 chromatography of urinary components. Elution of cytidine and inosine is monitored by absorbance at 254 nm, of ribose by phenyl sulphuric assay. For further details see text.

clear that other factors play a part in the retention and elution of the nucleosides. Changes in porosity between the various matrices due to different cross linking present may be one causative factor. Non-cyclic nucleotides (represented here by 5μmoles adenosine-5'-monophosphate, AMP) are shown to be eluted in both the sugar and nucleoside fraction and, given their basic properties, the catecholamines would also be eluted in the nucleoside fraction.

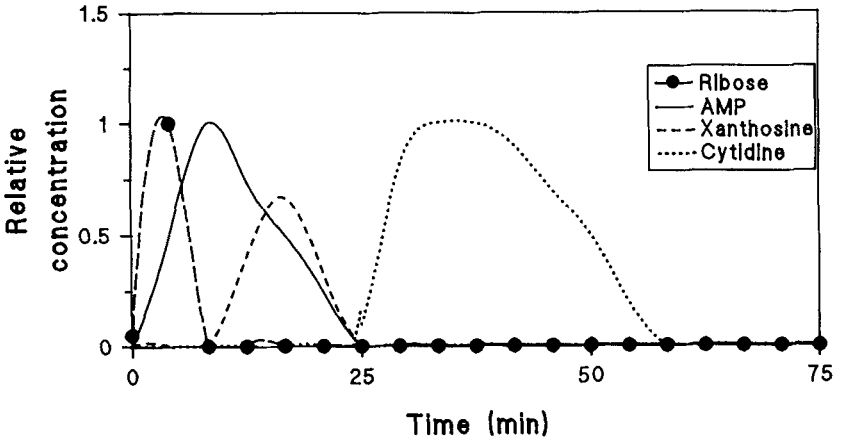

Figure 4. Dowex-50W chromatography of urinary components. Elution of cytidine, xanthosine and AMP is monitored by absorbance at 254 nm, of ribose by phenyl sulphuric assay. The elution of ascorbic acid and uridine, not shown on plot, is essentially identical to that of ribose. For further details see text.

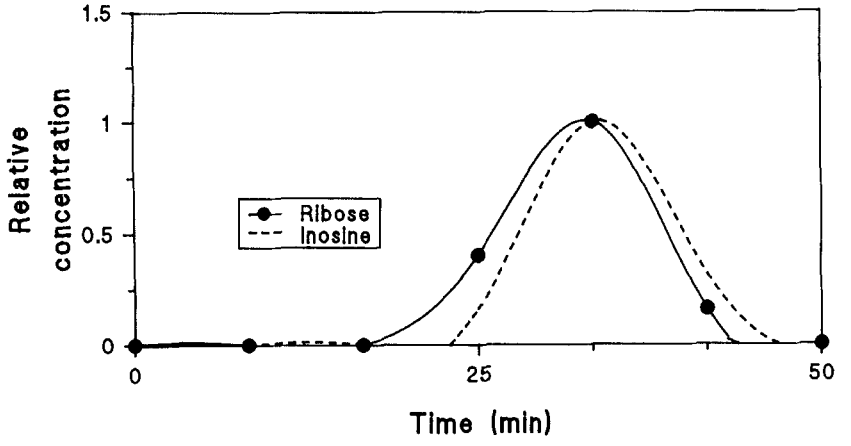

Figure 5. QAE-Sephadex (formate) chromatography of urinary components. Elution of inosine is monitored by absorbance at 254 nm, of ribose by phenyl sulphuric assay. For further details see text.

A QAE-Sephadex (formate form) column was prepared as described in the experimental section and the application of inosine (as a standard nucleoside) and ribose shows that no separation is achieved by the QAE column (Fig. 5), with other nucleosides exhibiting the same elution profile. Redgewell[12] had suggested preparing the QAE resin in the bicarbonate form as an alternative. This bicarbonate form (Fig. 6) separates less-basic nucleosides (for example uridine, xanthosine) from the vast majority

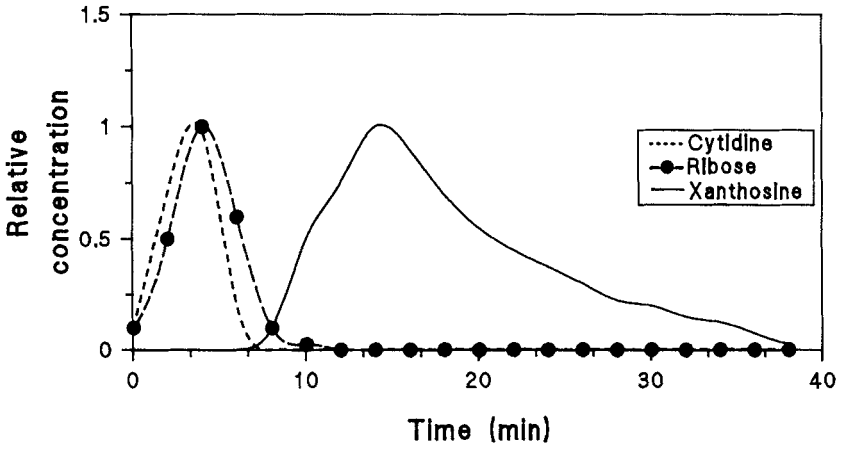

Figure 6. QAE-Sephadex (bicarbonate) chromatography of urinary components. Elution of cytidine and xanthosine is monitored by absorbance at 254 nm, of ribose by phenyl sulphuric assay. The elution of inosine and uridine, not shown on plot, is essentially identical to that of xanthosine; the elution of adenosine, not shown on plot, is essentially identical to that of cytidine. For further details see text.

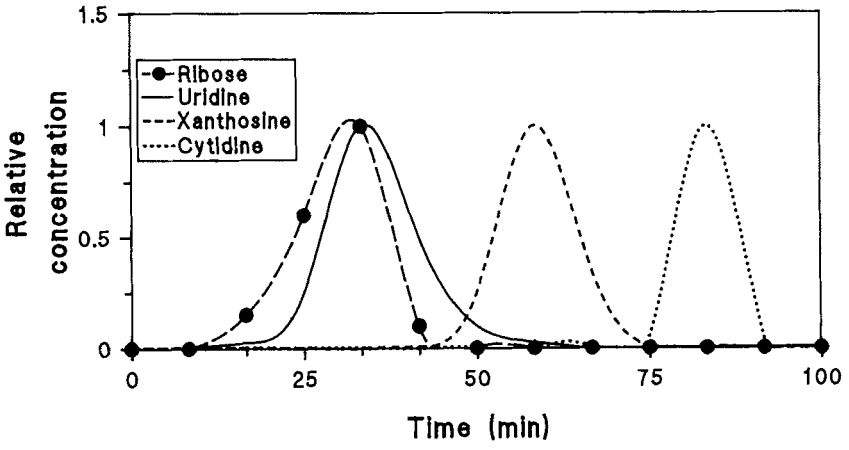

Figure 7. SP-Sephadex chromatography of urinary components. Elution of cytidine, uridine and xanthosine is monitored by absorbance at 254 nm, of ribose by phenyl sulphuric assay. For further details see text.

Table 2. Summary of separations obtained with individual resins.

Resin	Column dimensions (cm)	Eluting solvent	Flow rate (ml/min)	Fraction volume (ml)	Fraction contents
Affi-Gel 601	6 x 1	NH_4Ac 0.25M;	3	30	Non-cis-diol components
		Formic acid, 0.1M	3	50	Cis-diol components
Chelex-100	50 x 1	H_2O	1	72	Sugars and less basic nucleosides
Dowex-1	40 x 1	H_2O	1	100	More basic nucleosides, sugars, some less basic nucleosides
Dowex-50W	7 x 0.7	H_2O;	1	5	Sugars, ascorbic acid, some less-basic nucleosides
		NH_4formate, 1M	1	50	Most nucleosides, catecholamines, some nucleotides
QAE-Sephadex (formate)	53 x 1	H_2O	1	50	All components studied
QAE-Sephadex (bicarbonate)	14 x 0.7	H_2O	1	4.8	Sugars and more basic nucleosides
		$NaHCO_3$, 0.05M	1	24	Less basic nucleosides
SP-Sephadex	50 x 1	H_2O;	1	50	Sugars, some less basic nucleosides
		NH_4OH, 0.4M	3	150	Most nucleosides

of the sugar, providing a less-basic nucleoside fraction with a contaminant of sugars now low enough to be applicable to mass spectrometric analysis. More basic nucleosides (cytidine and adenosine) co-elute with the sugars.

An SP-Sephadex column was also prepared and its benefit in separating sugars and nucleosides tested (Fig. 7). The ribose was found to be eluted in the water fraction, with most nucleosides (*e.g.* xanthosine, inosine and cytidine) being retained and subsequently eluted in the 0.4M ammonium solution fraction. Uridine again is eluted in the sugar fraction, ostensibly behaving in the same manner as on the Dowex-50W resin as described above. The SP-Sephadex column therefore allows the separation of the sugar and most basic and less-basic nucleosides. The findings of these experiments are summarized in Table 2.

These data suggest three possible sequential protocols to separate the nucleosides from the sugar fraction. The first is the application of the cis-diol fraction from the Affi-gel 601 column separation

step, after concentrating by freeze-drying and redissolving in minimum volume of water, to a Chelex-100 column in order to separate the more-basic nucleosides from the sugars. The freeze-dried sugar and less-basic nucleoside fraction would then be applied to a QAE-Sephadex (bicarbonate form) column in order to separate the less-basic nucleosides from the majority of the sugar. The second possible separation procedure would be the use of the SP-Sephadex column in the place of the Chelex-100 column, while in the third option a Dowex-50W column replaces the Chelex-100 column.

All of the proposed separations require the use of the Affi-gel 601 and QAE-Sephadex (bicarbonate form) columns and differ only in the second step of the protocol, the separation of the more-basic nucleosides from the sugars. Of the methods of separation the use of the Dowex-50W column is the more efficient given its shorter running time, smaller fraction volumes, thereby reducing the time required to freeze-dry the fractions, and smaller column diameters, thereby allowing a smaller volume of resin to suffice. The other two resins require a larger amount of resin for each sample and produce larger fractions. Also Chelex-100 resin requires ammonium elution of the more-basic nucleosides with a consequence of some copper ion elution from the matrix into the nucleoside fraction. Such cations have the potential to form adducts with molecular ions in any mass spectra produced, therefore complicating the analysis of the nucleosides.

The first step employed in the purification was the removal of particulate matter by centrifugation at 3,000g for 10min. The supernatant was next acidified with 0.6M $HClO_4$ (2:1 v/v), neutralized with 0.5M KOH after 15min and a second centrifugation at 10,000g for 15min carried out to remove proteins and other heat-sensitive macromolecules from the urine together with the removal of $KClO_4$. The supernatant was freeze-dried and retained as the nucleoside fraction. After dissolving the fraction in a small volume (2-3ml) of 0.25M ammonium acetate (pH 8.8), the sample was then applied to a Affi-gel 601 column which was then eluted with 30ml ammonium acetate (yielding the non-cis-diol fraction), followed by 50ml 0.1M formic acid (yielding the cis-diol fraction).

This cis-diol fraction was then freeze-dried, redissolved in approximately 0.5ml water before application to a Dowex-50W column . This second column was then eluted with 5ml water (the sugar fraction) followed by 50ml ammonium formate (pH 4.86) giving a nucleoside fraction, containing most of the nucleosides. The only contaminants which will remain in this latter fraction are the catecholamines and some of the non-cyclic nucleotides, both of which occur in concentrations below that of the nucleosides in urine. The sugar fraction from the Dowex column (also containing some less-basic nucleosides) was freeze-dried, dissolved in water, then applied to a QAE-Sephadex (bicarbonate form) column. This third column produces a 4.8ml sugar fraction and a 24ml nucleoside fraction, the latter containing low enough sugar levels for mass spectrometric analysis.

The UV chromatographs obtained at each stage of this procedure after addition of standard compounds indicated that 100% of the nucleoside material recovered was in the retained fractions. To

quantitate recovery, a radiolabelled standard basic-nucleoside, adenosine (shown to be 96% pure, see exp. section) was added to individual stages of the procedure with 5ml patients urine and the cumulative yield calculated. The recoveries of the Affi-gel 601 formic acid fraction and the Dowex-50W ammonium formate fraction give a final yield of 92.5%. Recovery of four unlabelled nucleosides added to patients urine was also determined by quantitative mass spectrometry, giving recoveries of inosine 77.6% +/-12.9, dimethylguanosine 72.25% +/-12.25, pseudouridine 72.9% +/-8.4 and guanosine 81.5 +/-9.2. This high yield together with the efficient removal of contaminating urinary factors from the nucleoside fraction provides a useful routine procedure to clean up patient urine samples prior to MS analysis. For quantitative purposes the introduction of an internal standard to the urine sample, before it is applied to the Affi-gel 601 column, enables the monitoring of any unexpected loss of nucleosides in the purification procedure. For this purpose 50μg tubercidin is to be included in our further studies.

The protocol developed here is designed for application in our mass spectrometric analysis of cancer patients' urine. It provides a sequential, routine and reliable procedure to clean-up such samples which is not available by a single HPLC separation or on-line LC-MS system. The efficacy of the procedure can be seen from a comparison of a single-ion scan for cytidine in a urine sample that has not undergone this chromatographic process (Fig. 8a) and after chromatography (Fig. 8b). Scanning for *m/z* 244, the protonated molecule of cytidine, no clear cytidine-derived peak is apparent in the untreated urine sample, but a single, major, peak, which cochromatographs with standard cytidine with aretention time of 13.45 min, is apparent after the purification protocol. This procedure will enable the monitoring of such urinary nucleosides by electrospray MS without the need for derivatization, necessary in the GC-MS protocol employed previously[1]. As well as our purposes it should equally be of value in the analysis of nucleosides in samples in which they are minor components, in cell culture- or microbial growth media or mammalian tissue fluids in addition to urine.

Experimental Section

Standards. Standard compounds were obtained from Sigma (Poole, Dorset, U.K.) with the exception of: ammonium formate, ascorbic acid, copper chloride, ribose, ammonia solution, sodium formate, sodium hydroxide and perchloric acid (all from BDH Ltd, Poole, England). Chelex-100 and Affigel 601 were purchased from Biorad (Bio-Rad House, Maylands Ave., Hemel Hampstead, UK). Hydrochloric acid and sulphuric acid were purchased from Fisher Scientific (Loughborough, Leic. UK). Formic acid, ammonium sulphate and potassium hydroxide were purchased from Fisons (Loughborough, Leics, UK). [8-^{14}C]-adenosine was purchased from Amersham Ltd. and Pico-Fluor 40 was purchased from Canberra-Packard.

Sample Application. Samples of urine were obtained from Singleton Hospital Oncology Department and treated as described above. A Beckman J2-MC Centrifuge and an Edwards Modulyo Freeze-drier were used.

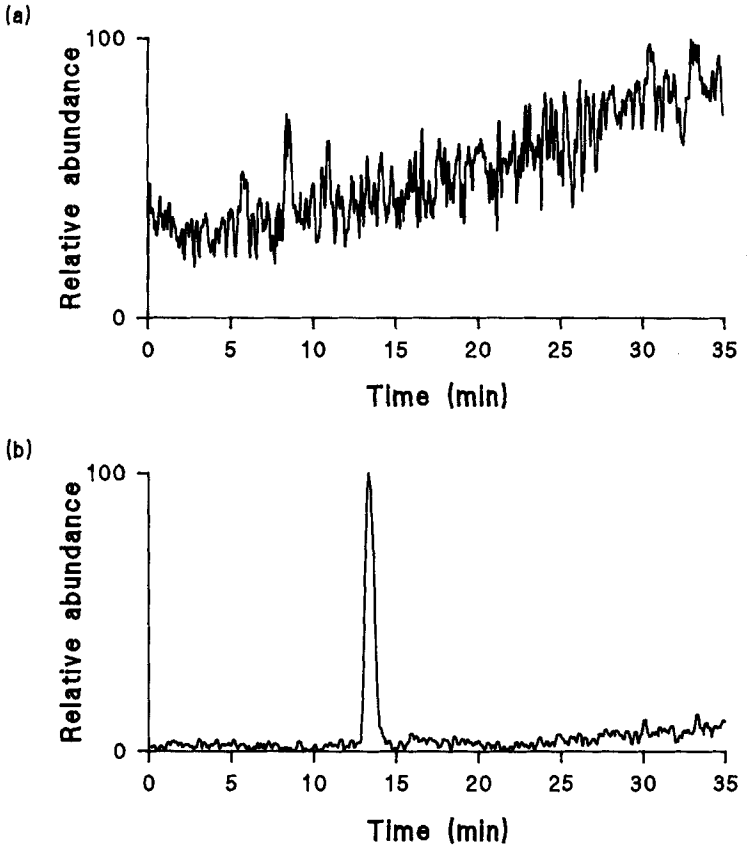

Figure 8. Electrospray mass spectrometric single-ion monitoring for *m/z* 244 (protonated molecule of cytidine) in (a) deproteinated urine sample, and (b) the same sample after chromatography by the method developed. For further details see text.

Column chromatography. Each of the chromatography columns were connected up to, and the elution flow rate maintained by, a EYELA Micro-tube pump MP-3 (Tokyo Rikakikai Co. Ltd.), with the exception of the Affi-Gel 601 column which was run on gravity flow. The eluent was diverted to a 8300 UVICORD II UV Spectrophotometer (LKB, Stockholm Bromma 1, Sweden), set to a wavelength of 254nm, which recorded the UV absorbance chromatograph on a PICO-LOG Data System (Pico Technology Ltd., Cambs, UK). This allowed the detection of most of the standard compounds added to the columns, with ribose being detected using the phenyl-sulfuric detection method[16]. After passage through the UV detector the eluent passed to a 2212 HELLIRAC Fraction Collector (LKB, Stockholm Bromma 1, Sweden) set to collect 1-2ml fractions. To 1ml of these fractions 5%phenol was added (1ml) followed by 5ml concentrated sulfuric acid. The colour was left to develop at 25-30°C

for 10 mins, and then the absorbance measured at 490nm in a Hewlett Packard 8452A Diode Array Spectrophotometer (Hewlett Packard). The elution profiles depicted in Figs 1-7 illustrate the distribution of components between fractions and are expressed as relative concentrations, *i.e.* as a fraction of largest UV absorbance or phenyl-sulfuric colour observed in any of the fractions with that specific compound.

Affi-gel 601: An Affi-gel 601 column of 6 x 1 cm was set up and equilibriated with 0.25M ammonium acetate (pH 8.8) and run at 3ml/min. To this column a compound without a cis-diol group (60μmol uracil) and a standard nucleoside (60μmol inosine) were added. The column was eluted with 30ml ammonium acetate (0.25M, pH 8.8), followed by 50ml 0.1M formic acid.

Chelex-100: Various column parameters of a Chelex-100 column (length, flow rate) were tested and the optimal separation was attained with a 50 x 1cm column eluted at 1ml/min with water. Standards of nucleosides (10 - 30μmol) and a representative of the major contaminants (10μmol ribose) were applied to this and subsequent columns.

Dowex: A 40 x 1cm Dowex-1 column eluted at 1ml/min with water and a 7 x 0.7cm Dowex-50W column eluted at 1ml/min with water for 5min and then 1M ammonium formate (pH 4.86) for 50min were used for optimum separation with these ion-exchangers. Standard samples of various nucleosides and ribose were added as with the Chelex-100 column, however the Dowex-50W column also had a non-cyclic nucleotide (5μmoles AMP) and ascorbic acid (10μmoles) added.

Sephadex ion-exchangers: After optimization of column parameters for sugar - nucleoside separation, a QAE-Sephadex (formate form) of 53 x 1cm was set up and eluted with water at 1ml/min; a shorter QAE-Sephadex (bicarbonate form) column was used (14 x 0.7cm) and eluted with water for 5min then 0.05M sodium bicarbonate for 40min at 0.6ml/min. An SP-Sephadex column of 50 x 1cm dimensions was shown to give the best sugar - nucleosides separation, elution with water for 50min (at a flow rate of 1ml/min) was required followed by a 0.4M ammonium solution at 3ml/min.

Radiolabelled standard addition. In order to determine the purity of the radiolabelled adenosine it was run on paper chromatography with a water solvent together with a separate standard of unlabelled adenosine. The unlabelled adenosine was detected using a UV light at 254nm and the radiolabelled side of the paper was counted on a Bioscan System 2000 Imaging Scanner. The radiolabelled adenosine was found to be 96% pure; 2.2 nmol [G-^{14}C]-adenosine (50,000dpm) was added to 5ml samples of patients urine. The columns were run as described above in the purification procedure and a 1ml sample of the fractions obtained added to 5ml of Pico-Fluor 40 and the radioactivity recorded on a Wallac 1217 Rackbeta Liquid Scintillation Counter (Wallac, Milton Keynes, UK), counting for 5min with a [^{14}C]- efficiency of 60%, in order to determine the yield.

Mass spectrometric determination of recovery. A mixture of 9.375μg each of inosine, dimethylguanosine, pseudouridine and guanosine were added to 5mL aliquots of urine samples. The

samples were then purified as described above and the level of these four nucleosides in spiked and untreated samples determined using a Finnegan Mat LCQ mass spectrometer in positive electrospray mode under standard operating conditions. The difference between the spectra, with the use of previously constructed calibration curves for quantitation of these four nucleosides, was used to determine recovery.

Resin preparation and regeneration. The Affi-Gel 601 was soaked in 0.25M ammonium acetate (pH 8.8) for 8-10 hours before use and between runs. Chelex-100 was prepared by soaking in 1M copper chloride for 8-10 hours followed by an 8-10 hour soaking in 1M ammonia solution, the resin was equilibriated in water before use. The chelex-100 was regenerated between runs by another soaking in 1M ammonia solution. The Dowex resins (both Dowex-1 and Dowex-50W) were prepared by soaking in 1M sodium hydroxide followed by soaking in 1M hydrochloric acid (all soakings lasting 8-10 hours), and equilibration in water (until giving a pH solution of 4.5-5) before use. Regeneration was performed by soaking in 1M hydrochloric acid. QAE-Sephadex was prepared in the formate form by soaking in 0.5M sodium formate (10g in 500ml), changing the sodium formate approximately 6 times a day; after 2 days the resin was stored in 0.05M sodium formate: before use the column was equilibriated with water. The bicarbonate form was prepared in the same way but using ammonium bicarbonate solutions and water equilibriation until pH 7.4-7.6. Regeneration of the QAE-Sephadex resins (both forms) was performed by an 8-10 hour soaking in the relevant 0.5M solution before storage in a 0.05M solution. SP-Sephadex was equlibriated in 2 volumes of 0.5M ammonium sulphate over 2 days, followed by filtration, resuspension in 7% formic acid for several hours and finally storage in 1% formic acid. The column was equilibriated with water to pH 4.5-5 before use and regenerated by performing the formic acid requiring sections of the preparation procedure.

Mass spectrometric analysis. LC-MS was carried out on a Hewlett-Packard 110 HPLC with a 5μm C18 column (150 x 4.6mm) linked to a Finnegan LCQ ion trap mass spectrometer, using an ammonium acetate/methanol gradient (0 - 30% over 35 min) at a flow rate of 0.2mL/min.

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