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TRACE ENRICHMENT OF PYRIMIDINE NUCLEOBASES, 5-FLUOROURACIL AND BROMACIL ON A SILVER-LOADED THIOL STATIONARY PHASE WITH ON-LINE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A silver-loaded thiol stationary phase was used for the trace enrichment of pyrimidine nucleobases (uracil, thymine and cytosine) and some related compounds (5-fluorouracil, bromacil, uridine). The critical parameters for the sorption of these compounds on Ag(I)–thiol are the pH value of the sample solution and the flow-rate with which they are flushed through the pre-column. Complete recovery for all compounds except uridine was obtained at pH 11.0 at a flow-rate of 0.6 ml/min. The desorption mechanism was based on protonation of the analytes. Efficient on-line desorption to the C_{18} analytical column was achieved by injecting a plug of 60 μl HNO$_3$ (pH 1.2) onto the Ag(I)-thiol pre-column. After separation the analytes were detected by UV detection at 269 nm. For 5-fluorouracil a detection limit of $3 \cdot 10^{-9}$ M was obtained for the preconcentration of 1.0 ml with a reproducibility of 2.3% ($n = 6, 5 \cdot 10^{-7}$ M). The column efficiency measured in terms of plate number for 5-fluorouracil was 11 000 for direct injections and 9000–10 000 for 1.0-ml preconcentrations, indicating that the desorption step causes no appreciable band broadening. Applications of the described method for the determination of 5-fluorouracil in plasma and for the herbicide bromacil in surface water are presented.

INTRODUCTION

Nucleobases are the primary building blocks of the genetic material. The five bases found in nucleic acids (Fig. 1) are heterocyclic aromatic compounds, either pyrimidines or purines, the latter containing a fused pyrimidine–imidazole ring system[1]. The two nitrogen atoms in the pyrimidine ring and the carbonyl and amino substituents contribute to shifts in electronic distribution resulting in lactim–lactam and amine–imine tautomerism[2] which is of fundamental importance to the structure and functioning of the nucleic acids. Although at physiological pH the stable lactam and amine forms predominate, the occurence of rare tautomeric forms has been implicated as a possible mechanism of spontaneous mutation[2].

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Lactam   Lactim

uracil : X = H   (pK 9.6)

thymine: X = CH$_3$ (pK 9.8)

5-fluorouracil: X = F (pK 7.9)

cytosine

pK 4.6

Amine   Imine
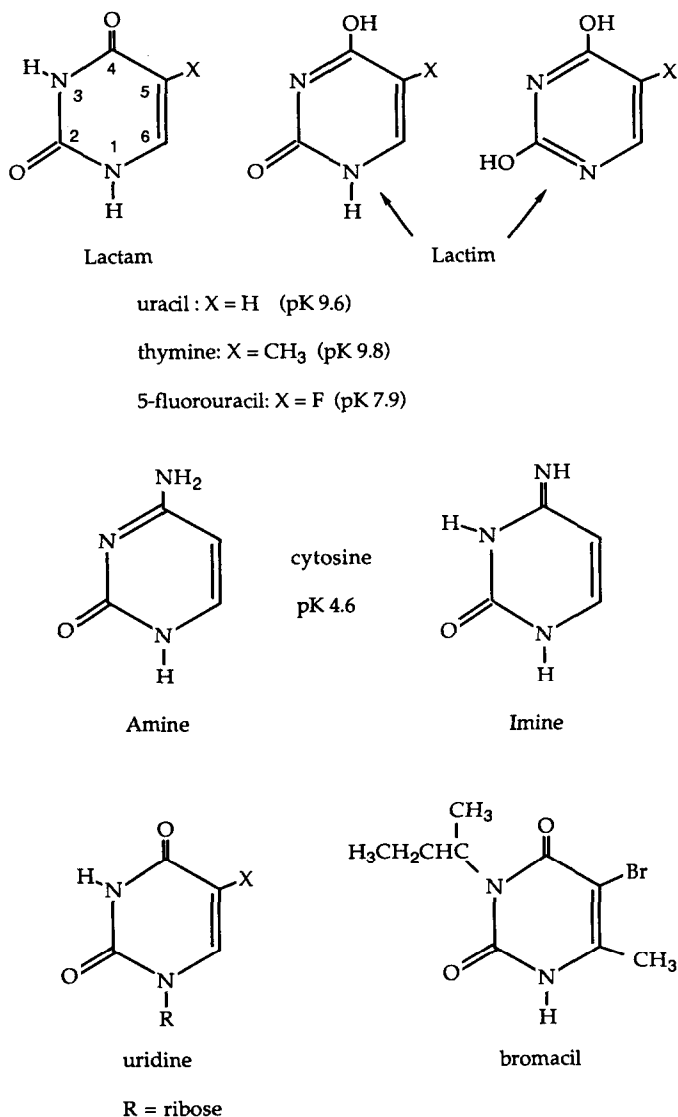
uridine   bromacil

R = ribose

Fig. 1. Structure of pyrimidine nucleobases and derivatives.

Certain, not naturally occurring bases such as 5-fluorouracil (FU) and 6-mer-captopurine are used as chemotherapeutic and mutagenic agents, as they are readily incorporated in nucleic acids instead of the natural bases[1]. FU has been used for nearly 25 years in the treatment of solid tumors, and pharmacokinetic studies on uptake, distribution and metabolism of FU have been carried out since then. Chro-matographic methods such as gas chromatography (GC) and liquid chromatography (LC) make accurate and sensitive analysis of FU and its metabolites possible. How-ever, FU is metabolized fairly rapidly after administration with an initial half life of

10–15 min, and within 3 h after bolus injection, the plasma concentration falls below $5 \cdot 10^{-8}$ M, $i.e.$, below the reported detection limit for most methods, including GC–electron impact mass spectrometry[3]. A limit of detection of $3 \cdot 10^{-9}$ M (0.39 ng/ml) was reported for electron-capture negative-ion mass spectrometry[4]. This method, however, requires derivatization of FU with an electron-capturing sensitive reagent, in addition to other sample pretreatment steps.

Samples of biological origin usually require a multi-step clean-up sequence, including deproteination and liquid–liquid extraction, to extract the compound(s) of interest prior to chromatographic analysis. Multiple liquid–liquid extractions, apart from being time consuming, often lead to losses in analyte recovery. Sample deproteination was conventionally carried out by precipitation with perchloric or trichloroacetic acid and neutralization with alkali or an amine-freon solution[5]. More recent techniques include ultrafiltration or clean-up on a C_{18} bonded silica stationary phase[6]. The use of a C_{18} phase offers the possibility of sample clean-up and trace enrichment of apolar and weakly polar compounds. C_{18} pre-columns are often used, on-line, in automated systems as disposable cartridges[7].

Biologically active compounds are usually weakly polar to polar and are therefore not effectively retained on a C_{18}-bonded silica phase. Frei et $al.$[7] and Nielen et $al.$[8] investigated the use of suitable sorbents such as ion exchangers and metal-loaded stationary phases for the on-line trace enrichment of polar organic compounds. Sorption on metal-loaded phases occurs via complexation and may therefore be applicable to the pyrimidine bases and their nucleosides and nucleotides which form complexes with a variety of metal ions[9,10]. Metal ions have been used in LC in various ways.

(i) As complexing agents in the mobile phase: Ag(I) was used in the separation of retinyl esters[11] and a number of heterocyclic and unsaturated compounds[12]. Mg (II) was used for the separation of nucleotides on a dithiocarbamate column[13].

(ii) Immobilized on a chelating stationary phase: Cu(II) was loaded on a silica-polyol phase for the separation of pyrimidine bases and nucleosides[14]. Retention was proposed to take place via a combination of ligand-exchange, reversed-phase and adsorption mechanisms. Stationary phases developed for such systems were of the iminodiacetate silica-polyol or 8-hydroxyquinoline (oxine) silica-polyol type.

In the present study we investigated experimental parameters affecting the sorption of FU, uracil, uridine, thymine and cytosine on a Ag(I)–thiol stationary phase as well as conditions affecting on-line desorption from the stationary phase to the analytical column. This method was applied to the determination of FU in plasma, and of the herbicide bromacil, a uracil derivative, in surface and tap water.

EXPERIMENTAL

Apparatus

The LC system (Fig. 2) consisted of three laboratory-made six-port Valco-type injection valves, a 1.0-ml sample loop, a 60-μl loop to contain HNO_3 (pH 1.2) needed for the desorption of analytes, a 10.0 × 4.0 mm laboratory-made stainless-steel precolumn and holder (Chrompack, Middelburg, The Netherlands) and 200 × 4.6 mm I.D. Hypersil ODS 5-μm (Shandon Southern, Runcorn, U.K.) analytical column. The carrier solution for preconcentration was delivered by a Gilson (Villiers le Bel, France) Model 302 single-head reciprocating pump and the LC eluent by a Waters
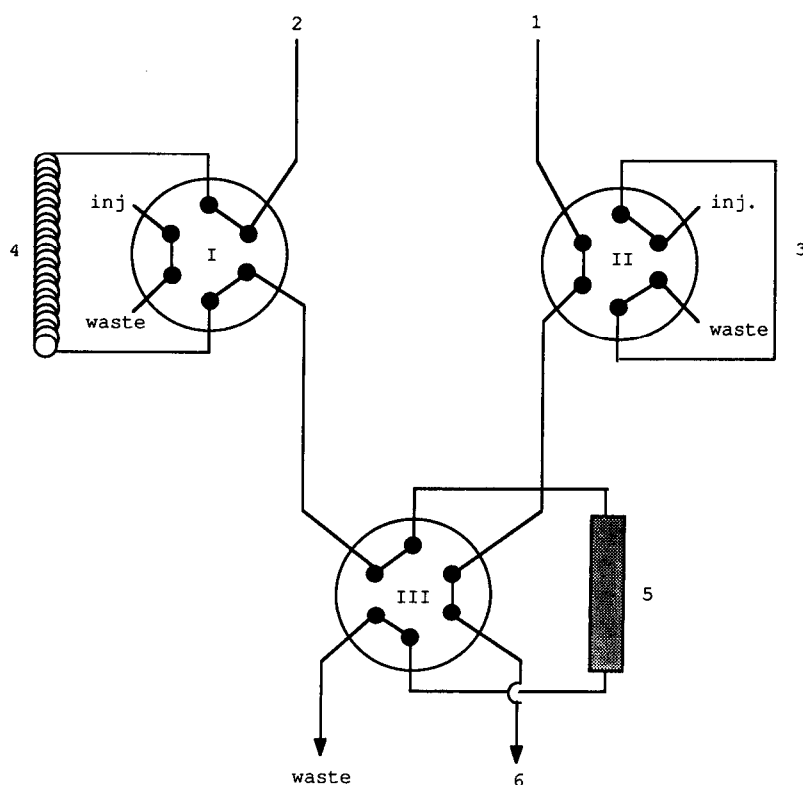
Fig. 2. Scheme of the preconcentration system. 1, LC pump; 2, preconcentration pump; 3, 60-μl injection loop for HNO_3; 4, sample injection loop (1.0 ml); 5, Ag(I)–thiol pre-column; 6, to analytical column/detector.

(Milford, MA, U.S.A.) Model 510 dual-head reciprocating pump. Pulse dampers are incorporated into the system for each pump. The LC eluent was aqueous 2 mM acetate buffer (pH 5.8) containing 0.1 mM cetyltrimethylammonium bromide (CTAB). Two variable-wavelength UV detectors, a Pye Unicam LC-3 UV (Philips, Eindhoven, The Netherlands) and a Kratos (Ramsey, NJ, U.S.A.) Spectroflow 757, were used for detection at 269 nm.

Chemicals and reagents

Uracil, thymine, sodium N,N-diethyldithiocarbamate (DTC) and CTAB were obtained from Merck (Darmstadt, F.R.G.), 5-fluorouracil, 5-chlorouracil and uridine from Janssen (Beerse, Belgium), and cytosine from Sigma (St. Louis, MO, U.S.A.). Standard solutions of 10^{-3} M were prepared from these compounds in distilled, deionized water.

A standard solution of bromacil (gift of A. de Kok, Rijkskeuringsdienst van Waren, Alkmaar, The Netherlands) of 300 μg/ml was prepared in HPLC-grade methanol (Baker, Deventer, The Netherlands). All other chemicals used were Baker analyzed reagents.

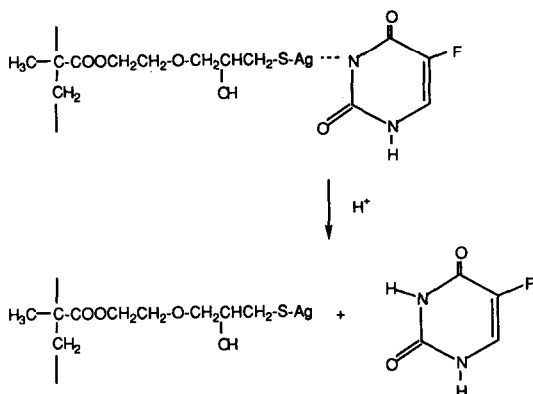Fig. 3. Desorption of FU from the Ag(I)–thiol stationary phase.

Trace enrichment and chromatography

A 40–63 μm thiol-modified hydroxyalkyl-methacrylate gel, Spheron Thiol 1000 (Lachema, Brno, Czechoslovakia) stationary phase (Fig. 3) for trace enrichment was slurry packed via a 5 ml syringe into a stainless-steel pre-column, loaded off-line with 5 ml 10 mM AgNO$_3$ and flushed with 5 ml deionized, distilled water and 5 ml HPLC-grade methanol. The preconcentration carrier solution was aqueous NaOH (pH 10.5). Equilibration of the silver-loaded stationary phase was carried out on-line by flushing with the carrier solution for 10–15 min.

A 1.0 ml sample plug containing $5 \cdot 10^{-5}$–10^{-8} M of various combinations of model compounds was loaded onto the pre-column at a carrier flow-rate of 0.6 ml/min for 4.0–4.5 min (Fig. 2). On-line desorption to the analytical column was carried out by injecting a plug of 60 μl HNO$_3$ (pH 1.2) into the LC eluent stream (valve II) and switching the eluent flow (valve III) to the pre-column to desorb the preconcentrated analytes and transfer them to the analytical column. If a DTC–CTAB ion-pair was used as a displacer an additional C$_{18}$ pre-column was inserted between the Ag(I)-thiol pre-column and the analytical column in order to remove the excess of DTC. Next, the analytes were separated under isocratic conditions at a flow-rate of 1.2 ml/min. The analytical column was allowed to equilibrate with LC eluent overnight at a flow-rate of 0.1 ml/min to ensure stable operating conditions. Pure acetonitrile was used periodically to purge the column.

Direct injections (12 μl) of $5 \cdot 10^{-5}$ M standard solutions of the five model compounds, FU, uracil, thymine, cytosine and uridine, were run daily to test the analytical column performance. Direct injections of 60 μl of the same compounds were run for recovery calculations. Direct injections were made by replacing the Ag(I)-thiol pre-column by the appropriate sample loop in order to measure peak areas under the same conditions of extra-column band broadening.

Determination of bromacil in surface water

A stock solution of bromacil was prepared in HPLC-grade methanol. The carrier solution used for preconcentration was methanol–water (50:50), adjusted to pH 10.5 with 5 M NaOH. The LC eluent was methanol–acetate buffer (10 mM, pH

6.0) (50:50) containing 0.1 mM CTAB. Surface water was collected from a nearby canal, adjusted to pH 10.5, filtered through a 0.2-μm disposable membrane filter and spiked to the desired concentration. Samples of 3–12 ml were preconcentrated according to the procedure described above.

Determination of FU in human plasma

Two different approaches were used.

(i) Non-deproteinized plasma was either filtered through a 0.2-μm disposable membrane filter or centrifuged for 15 min at 1000 g to remove suspended and precipitated matter. After adjusting the pH to 11.5 the supernatant was introduced into the preconcentration system. A second pre-column (60.0 × 4.0 mm I.D.) packed with 10-μm PLRP-S (Polymer Laboratories, Church Stratton, U.K.) polymer phase was placed before the Ag(I)-thiol pre-column in order to remove macromolecular and apolar components prior to trace enrichment.

(ii) For deproteination, equal volumes of plasma and methanol were mixed and vortexed, then centrifuged for 20–25 min and the supernatant (methanol–water, 50:50) removed and adjusted to pH 10.5. Trace enrichment of deproteinized plasma proceeded without the need for an additional PLRP-S pre-column. Plasma was spiked to the desired concentrations prior to deproteination.

RESULTS AND DISCUSSION

Fundamental aspects

Tautomerism exhibited by pyrimidine nucleobases governs the acid–base and complexation behaviour of these compounds. Complexation with metal ions occurs via a Lewis-base type interaction at the electron-donating sites in the purine and pyrimidine ring. The nature of the donor atom is determined by the coordinated metal ion[9]. Uracil and its 5-halogenated derivatives form strong complexes with Ag(I)[15], Pd(II)[16] and a number of divalent metal ions[17,18]. The order of stability constants for the FU complexes decreases as Ag(I) (4.9 · 10³) > Cu(II) (1.23 · 10³) > Ni(II) (28.3). A 1:1 as well as a 1:2 complex between Ag(I) and FU has been suggested[17,18].

Complexation with a metal ion takes place at a deprotonated site (see Fig. 1) and is therefore strongly pH dependent. Dissociation takes place at N(3) (pK_{a1} = 7.9)[9] making the lone electron pair of the nitrogen available for complexation via a Lewis-base interaction. At elevated pH further deprotonation takes place at N(1) (pK_{a2} = 13)[9].

Sorption of pyrimidine nucleobases on a metal-loaded pre-column on-line to the analytical system was investigated in the present study. Spheron-thiol as stationary phase and Ag(I) as the complexing ion were chosen for the following reasons. (i) The stability constant for the Ag(I)–FU complex is high enough to form stable complexes but complexation is not irreversible; (ii) Ag(I) prefers nitrogen to oxygen as electron donor[9]; (iii) Ag(I) forms π–π bonds with the thiol group, which approach covalent bond strength (*e.g.*, log k_1 of Ag(I)–mercaptoethanol is 13.0[19]; (iv) the polymeric thiol-phase is stable over a wide pH range (1–13) and is therefore not subject to deterioration during prolonged use at high pH.

Several strategies can be employed to desorb the analyte from the stationary phase.

(a) A compound forming stronger complexes with the metal can displace the analyte. Nielen et al.[8] used cysteine to displace 2-mercaptobenzimidazole from Hg (II)-loaded phases. We have initially tried diethylthiocarbamate (DTC) ions, which form strong complexes with silver, to displace FU (see Fig. 6c). Before reaching the analytical column, however, excess DTC must be trapped on another pre-column, otherwise a strong DTC signal can interfere with the detection of the analytes. Excess DTC can be trapped on a C_{18} precolumn prior to reaching the analytical column by the addition of CTAB which forms a strongly apolar ion-pair with DTC.

(b) A metal ion can desorb the analyte by complexation with the analyte itself (I) or with the thiol group of the stationary phase (II). In both cases the analytes are eluted to the analytical column as metal complexes and must be chromatographed as such.

(c) Desorption can take place via protonation. Veuthey et al.[20] used a low pH in the LC mobile phase in order to desorb amino acids bound to a Cu(II)-loaded dithio-carbamate stationary phase.

In contrast to a and b(II), where the thiol stationary phase must be regenerated after every preconcentration, method c offers two advantages:

(i) Protonation with a strong acid such as HNO_3 converts the pre-column to the free Ag(I)–thiol form which is then immediately reusable after desorption without any further treatment. Due to the high stability of the Ag(I)–thiol bond, Ag(I) is not stripped off during analyte desorption. Therefore the use of the thiol stationary phase is favored over oxine[8,9] which forms much weaker complexes with Ag(I) (log k_1 for Ag–8-hydroxyquinoline is 6.6)[19].

(ii) Excess displacer does not interfere with the detection system, i.e., a pre-column for removal of the displacer is not necessary.

Method c was therefore chosen to desorb the uracil compounds from the stationary phase (Fig. 3).

Influence of pH and flow-rate on sorption

Breakthrough experiments have shown that the pH value of the sample solution and the flow-rate of the carrier solution with which the sample is loaded onto the stationary phase are the critical parameters which determine the sorption of the model compound, FU, on the Ag(I)–thiol column. Breakthrough of FU is mainly determined by complexation kinetics and thermodynamics and not by the capacity of the Ag(I)–thiol column. At pH 9 breakthrough occurs almost immediately, while at pH 10.8 FU is completely retained over a long time interval. At a carrier solution flow-rate of 0.4 ml/min the breakthrough volume for FU was over 15 ml.

Experiments in the preconcentration mode were carried out to determine analyte recovery as a function of the carrier solution flow-rate and of the pH value of the sample solution using a carrier solution of pH 10.5. As an example, percentage recovery as a function of carrier flow-rate at sample pH 11.0 is shown in Fig. 4 for five test compounds. Noticeable losses start to occur for flow-rates of over 0.7 ml/min. From other experiments (data not shown) it became evident that analyte recovery markedly decreased for a sample pH of 10.4, and was down to 30–40% for pH 9.9. At sample pH values of over 11, FU, uracil, cytosine and thymine consistently showed complete recovery. The relatively large losses still observed for uridine under these conditions indicate that steric hinderance prevents efficient adsorption.
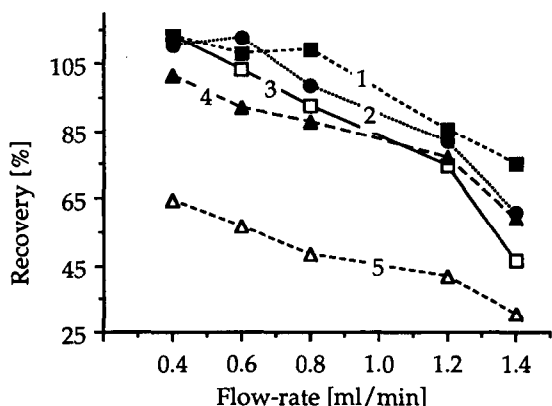
Fig. 4. Percentage recovery of FU (1), uracil (2), cytosine (3), thymine (4) and uridine (5) at pH 11.0 as a function of the carrier flow-rate.

Percentage recovery of FU *versus* carrier flow rate at four pH values is presented in Fig. 5. A flow-rate of 0.6 ml/min, a carrier solution pH of 10.5 and a sample pH of 11.0 were chosen as optimum conditions for the preconcentration of this and other uracil derivatives on the Ag(I)–thiol phase.

Trace enrichment from sample solutions containing 50% methanol also resulted in complete recovery of the analytes under the same conditions as specified above. This indicates that no hydrophobic interactions with the methylmethacrylate backbone of the Ag(I)-thiol phase were involved in the sorption process.

Influence of pH and displacer volume on desorption

At neutral pH the pyrimidine bases are fully protonated. Therefore it should be possible to desorb the analytes from the Ag(I)–thiol phase by flushing the pre-column
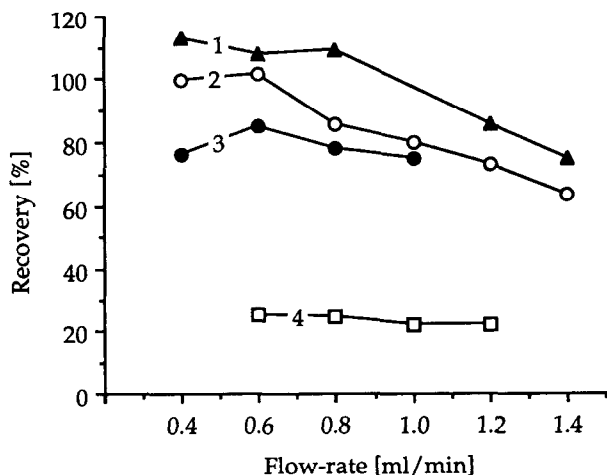

Fig. 5. Percentage recovery of FU at pH 9.9–12.2 as a function of the carrier flow-rate. 1, pH 12.1; 2, pH 11.0; 3, pH 10.4; 4, pH 9.9.
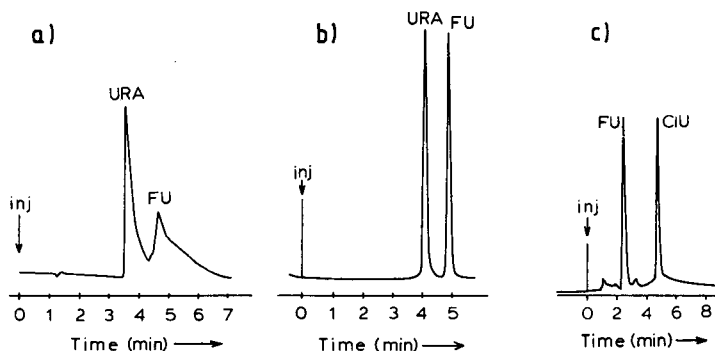
Fig. 6. On-line desorption of the analytes to the analytical column (a) by flushing the Ag(I)–thiol column with the LC mobile phase (URA = uracil), (b) by injecting 60 μl HNO$_3$ (pH1.2), (c) by injecting 340 μl DTC–CTAB (1 mM, pH 6.0 adjusted with 10 mM acetate buffer) (CU = 5-chlorouracil). Conditions: 5 · 10^{-6} M of analytes (preconcentration of 1.0 ml); carrier flow-rate 0.6 ml/min; pH of the sample solution: 11.0; LC conditions: see Experimental.

with, for example, the LC eluent which has a pH of 5.8. However, slow desorption kinetics at pH 5.8 resulted in considerable peak broadening and asymmetry as shown in Fig. 6a. A lower pH value is necessary for efficient desorption and therefore plugs of HNO$_3$ were injected into the LC eluent (see Fig. 2). The use of 0.01 M HNO$_3$ resulted in improved peak shapes and optimum results were obtained at pH 1.2 (Fig. 6b).

The volume of the displacer solution is also critical in determining the desorption efficiency. The displayer volume should be large enough to efficiently desorb the analytes from the pre-column, but care should be taken not to introduce volumes too large that cause deterioration of analytical column performance. Starting with a nitric acid volume of 340 μl we could reduce this to 60 μl without loss of efficiency. Using larger volumes of higher pH resulted in poor peak shapes. This indicates that a small volume of nitric acid of low pH produces a narrow elution zone in which the sorbed analytes can be rapidly and efficiently protonated, resulting in a favorable elution profile (Fig. 6b). For means of comparison a chromatogram of FU and 5-chlorouracil obtained by the use of DTC–CTAB as a displacer (plug injection of 340 μl) is shown in Fig. 6c. While peak shapes and recoveries of the test components are similar to those obtained with protonation, the latter method has the disadvantage that an additional C$_{18}$ pre-column is required for the removal of the excess of DTC-CTAB and that the Ag(I)–thiol pre-column must be regenerated after each preconcentration step.

In all subsequent experiments desorption of the analytes was performed by injecting a 60-μl plug of nitric acid (pH 1.2). Chromatograms of a 12-μl direct injection (concentration of the model compounds: 5 · 10^{-5} M) and after preconcentration and desorption of a 1.0-sample of the model compounds at a concentration of 5 · 10^{-7} M are shown in Fig. 7 (for the lower uridine recovery, see Fig. 3). Column efficiency measured in terms of plate number for FU was compared for direct injections and for 1.0 ml preconcentrations and showed only a slight decrease: 9000–10 000 for preconcentration vs. 11 000 for direct injection, indicating that the de-
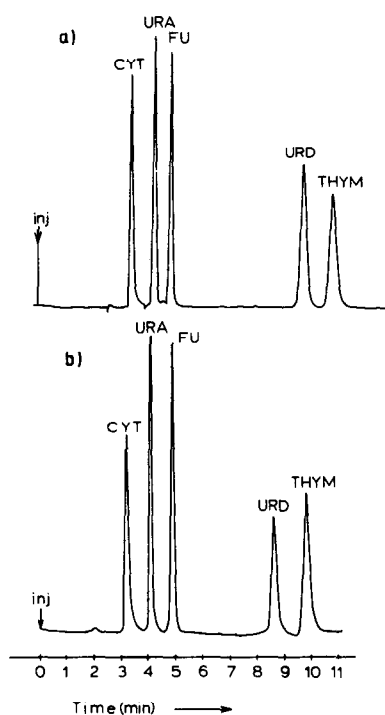
Fig. 7. Direct injection (12 μl, 5 · 10^{-5} *M*) and preconcentration (1.0 ml, 5 · 10^{-7} *M*) of cytosine (CYT), uracil (URA), FU, uridine (URD) and thymine (THY). Conditions: carrier flow-rate, 0.6 ml/min, pH of the sample solution, 11.0 (preconcentration), 5.8 (direct injections); attenuation, 0.02 a.u.f.s.; LC conditions, see Experimental.

sorption step causes *no* appreciable band broadening. The reproducibility for the preconcentration of 5 · 10^{-7} *M* FU was 2.3% (*n* = 6).

Regeneration of the pre-column packing

Veuthey *et al.*[20] eluted amino and carboxylic acids from a Cu(II)–dithiocarbamate stationary phase via protonation by flushing with the LC eluent of pH 2. Similarly, for the pyrimidine nucleobases desorption via analyte protonation leaves the silver bound to the thiol ligand and the Ag(I)–thiol phase can be continuously reused. Each desorption step is at the same time a pre-column regeneration step. Although we found that when working with standards the Ag(I)–thiol pre-column can be used over a period of several days before noticeable deterioration of sorption occurs, it is advisable to pack a new pre-column daily, especially when complex sample matrices are introduced into the system.

Chromatography and detection

The capacity factors of the uracil derivatives are very small on a C_{18} analytical column with pure water as eluent. This system is therefore not well suited for the analysis of complex samples. We found that adding CTAB to the mobile phase, although not influencing the *k'* values of cytosine and uracil, produced excellent

separation and peak shapes of these compounds, whereas elution of uridine and thymine was retarded. For cytosine and uracil retention is not governed by an ion-pairing mechanism; the addition of CTAB serves mainly to cover residual silanol groups of the C_{18} phase, thereby reducing the tailing of polar compounds. In the case of FU, a small fraction of which is deprotonated at pH 5.8 ($pK_a = 7.6$), ion-pairing with CTAB may contribute to the overall retention mechanism which is indicated by higher capacity factors at increasing CTAB concentrations. Peters et al.[21] used ion-pairing chromatography of the anion forms of the uracil derivatives at high pH on a hydrophobic PRP-1 polymer phase; however, such packing material will not give as high a column efficiency as a C_{18} column of the same length.

Both variable-wavelength UV detectors showed excellent linearity over three orders of magnitude in the concentration range of interest ($10^{-6} - 10^{-8}$ M) with a correlation coefficient of 0.9998. Using the Kratos Spectroflow 757 a detection limit for FU of 0.4 ng ($3 \cdot 10^{-9}$ M for the preconcentration of 1.0 ml) was reached at 269 nm.

In order to increase the selectivity of the analytical system fluorescence detection was investigated. The fluorescence of FU and thymine is strongly pH-dependent and reaches maximum intensity at pH 12 (ref. 22). Achieving this pH value required post-column addition of NaOH (pH 12.1) via a mixing piece. From all compounds investigated only FU and thymine yielded a fluorescence signal. The detection limit was $2 \cdot 10^{-7}$ M, or 26 ng FU which is almost two orders of magnitude higher than UV detection; therefore the latter method is favoured.

Applications

Determination of 5-fluorouracil in human plasma. The determination of FU in a biological matrix is an important application of on-line enrichment on a silver-loaded stationary phase. We therefore wanted to demonstrate that FU can be determined in, for example, human plasma. Deproteinized as well as non-deproteinized plasma samples were preconcentrated.

With non-deproteinized samples an additional PLRP pre-column was inserted between valves I and III (see Fig. 2) in the trace-enrichment scheme to trap apolar compounds. The blank plasma samples, however, gave rise to irregular interferences in the baseline as well as several large peaks eluting after approximately 13 min, indicating that the PLRP pre-column was not efficient enough in trapping interfering plasma components.

As an alternative, the plasma was deproteinized off-line prior to trace enrichment. Methanol was selected to this end, because its use is not accompanied by the drastic pH lowering which accompanies precipitation with trichloroacetic acid. Precipitation was carried out at neutral pH, the supernatant was adjusted to pH 11.0, and the sample was then introduced into the preconcentration system. Chromatograms of blank and spiked plasma samples after trace enrichment of 0.5-ml volumes are shown in Fig. 8. Even at the lowest detector attenuation setting, the plasma blank showed a rather undisturbed baseline in the region where the pyrimidine bases elute and FU concentrations of $2.5 \cdot 10^{-7}$ M could be determined in spiked plasma with an estimated detection limit of 10^{-7} M. Several later eluting (> 25 min) non-identified peaks most likely represent other compounds containing the pyrimidine ring such as nucleotides and polar plasma constituents which are also sorbed on the Ag(I)–thiol phase.
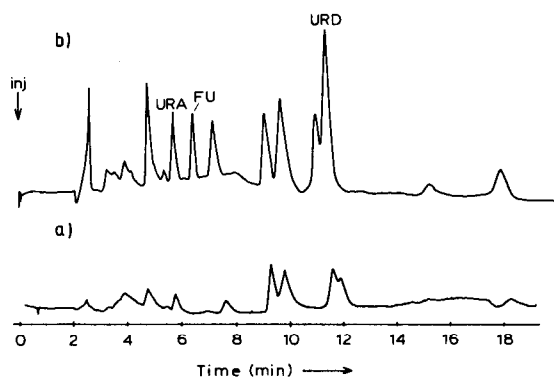
Fig. 8. Determination of FU in plasma (a) plasma blank; attenuation, 0.04 a.u.f.s. (b) Plasma spiked with $2.5 \cdot 10^{-7}$ M FU; attenuation, 0.01 a.u.f.s. Conditions: 0.5 ml plasma deproteinized with an equal volume of methanol, pH of the sample solution, 11.0; carrier flow-rate, 0.6 ml/min; LC conditions, see Experimental.

Determination of bromacil in surface water. The herbicide bromacil can be determined in surface and tap water by the above described method. Bromacil is structurally related to the parent compound uracil (see Fig. 1), but the N-3 ring position is not free for complexation to silver as it is occupied by an isobutyl group. Complexation can only occur at the N-1 position which is still subject to keto–enol tautomerism with the carbonyl oxygen. The question arose whether the minimum structure required for effective complexation with silver as a single $H-N-C=O$ ring moiety.

Under the optimum operating conditions (described in Experimental) we were
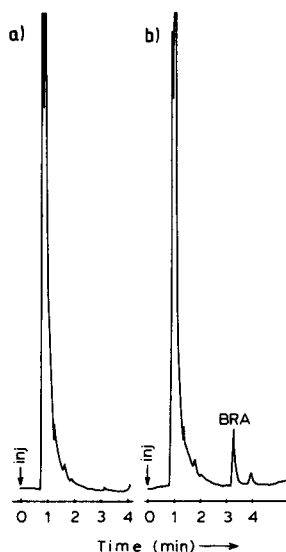

Fig. 9. Determination of bromacil in surface water. (a) Surface water blank; (b) surface water spiked with 15 ppb bromacil. Conditions: preconcentration of 3 ml; pH of the sample solution, 11.0; carrier flow-rate, 0.6 ml/min; attenuation, 0.01 a.u.f.s.; LC conditions, see Experimental.

able to preconcentrate bromacil on the Ag(I)–thiol stationary phase in the low-ppb range. Chromatograms obtained after preconcentrating 3.0 ml volumes of blank surface water spiked to 15 ppb are shown in Fig. 9. The detection limit for bromacil was 1.5 ppb (4.5 ng) for the preconcentration of 3-ml sample volumes. Sample volumes of up to 12 ml can be preconcentrated without breakthrough. With this method it is possible to determine low-ppb amounts of bromacil in water samples without the need for tedious and time-consuming sample pretreatment. Ease of automation of this method will enable large sample throughput.

CONCLUSIONS

A Ag(I)–loaded thiol stationary phase shows excellent properties as sorbent for pyrimidine nucleobases and their derivatives. The sorption is governed by complexation of the pyrimidines with the silver ion, and is strongly pH dependent. Desorption proceeds via a simple protonation mechanism. A small volume of strong acid suffices to elute the analytes to the analytical column in a narrow band and plate numbers for FU were consistently in the order of 10 000 compared to 11 000 for direct injections.

On-line trace enrichment and LC determination of pyrimidine nucleobases and their derivatives was possible with a detection limit of $3 \cdot 10^{-9}$ M for standard solutions and $1 \cdot 10^{-7}$ M for FU in plasma using UV detection. Further optimization of the clean-up process may lead to even lower detection limits. The experimental system can be easily automated, which is desirable for routine monitoring.

Metal-loaded stationary phases offer, from the applications side, a means to preconcentrate moderately polar to polar compounds and can therefore be an important tool in the routine determination of these compounds in environmental and biological matrices[8,23]. It has thus far not been possible to succesfully preconcentrate these classes of compounds on other types of sorbents. Further investigation in the use of metal-loaded stationary phases for on-line trace enrichment may extend the method to a broad spectrum of polar heterocyclic aromatic compounds that show properties similar to the pyrimidine bases. These investigations will initially have to focus on the fundamental aspects of metal–analyte interaction and the displacement of the analyte from a metal-loaded phase. The choice of metal will depend on the type of analyte(s), their acid-base behaviour, and the nature of the electron donor (N or O donor). In larger molecules, for instance the purine compounds, steric factors may play a role in the complexationg process in view of the heterogeneous interaction on the solid surface of the stationary phase. The central question of whether a "minimum" structure exists, which comprizes one or more functional groups and a favorable electronic distribution within the molecule, and is responsible for the desired complexation characteristics, is currently under investigation.

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