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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BIOLOGICALLY IMPORTANT PYRIMIDINE DERIVATIVES WITH ULTRAVIOLET—VOLTAMMETRIC—POLAROGRAPHIC DETECTION

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SUMMARY

High-performance liquid chromatographic separation of a number of biologically important pyrimidine derivatives was studied in the reversed-phase system. Good results were obtained using a C_{18} alkyl-bonded silica column and an aqueous citrate—phosphate mobile phase of pH 3.5. All eluted components are detected with the UV absorbance detector at 254 nm, whereas the voltammetric detector with a polymeric carbon-paste electrode detects only derivatives containing oxidizable functional groups (amino, mercapto) and the polarographic detector with a mercury electrode only those with reducible groups (nitro, aza). The signal of the electrochemical detectors is proportional to the number of electroactive groups in the solute molecule. The use of two or three detectors in series thus improves the resolution of complex mixtures and facilitates identification.

INTRODUCTION

Pyrimidine derivatives, especially uracil, cytosine and thymine, belong among the biologically most important substances. Many other derivatives, e.g. fluoro- or aza-, are contained in pharmaceuticals and are monitored from the point of view of their metabolism in living organisms. For these reasons, much attention has been devoted to their analysis.

Gas chromatography (GC) has often been used for the separation and determination of pyrimidine bases (e.g. refs. 1—4). However, these determinations require derivatization and thus high-performance liquid chromatography (HPLC) is more advantageous, permitting direct and rapid analyses that are also sometimes more sensitive (for example, the HPLC determination of 5-fluorouracil in plasma is twenty times more sensitive than the GC determination [5]). For a detailed survey of HPLC of pyrimidine derivatives see the review [6].

As pyrimidine derivatives contain functional groups of various properties in a

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single molecule (basic and acidic groups, groupings of various polarities), a great variety of chromatographic systems can be employed for their separation. Ion-exchange has been used for HPLC separation of pyrimidine and purine bases, both on cation [7—9] and anion [10—13] exchangers, with isocratic or gradient elution. For an anion-exchange separation, the effect of the composition and pH of the mobile phase and of the type and concentration of the counter-ion on the retention behaviour has been studied [9—11]. Separations on silica gel, using a non-polar mobile phase (dichloromethane—methanol—aqueous salt solution) [14—16], and on a macroporous styrene—divinylbenzene copolymer [17] have also been described.

A great contribution to the analysis of pyrimidines was the introduction of reversed-phase systems (e.g. refs. 18—30). Various chemically bonded phases have been employed. A comparative study [31] has shown that the best results are obtained with the Spherisorb ODS-2 phase. Pyrimidine and purine bases have been determined in serum and plasma (e.g. refs. 18, 21, 22, 25, 29), in hydrolysates of nucleic acids (e.g. ref. 20), with isocratic [31] or gradient [18—21, 32] elution. As counter-ions, dodecyl sulphate [27], 11-aminoundecanoic acid [26] and several quaternary ammonium salts [23, 33] have been used. The effect of the temperature, pH and the composition of the mobile phase, and of the counter-ion concentration on the separation has been studied [23, 27, 28, 30].

UV photometric and sometimes also fluorescence [21] detection has been used. For identification of pyrimidines and purines, the ratio of the absorbances at 254 and 280 nm, or a combination of the UV photometric and fluorescence detectors have been employed [18, 21].

The papers published so far deal almost exclusively with nucleobases and 5-fluorouracil. As a number of other derivatives may be encountered in the production of pharmaceuticals and in the study of their chemical reactions and metabolism, we studied the conditions for separation of a number of derivatives containing a great variety of functional groups. In separations of complex mixtures occurring mainly in biological samples, difficulties may arise concerning the resolution and identification of the components [18]. Therefore, we investigated the combination of UV detection with electrochemical detection, as the latter is characterized by considerable selectivity (e.g. ref. 34).

EXPERIMENTAL

Apparatus

An LC-XP liquid chromatograph (Pye Unicam, Cambridge, Great Britain) with a Separon C_{18} column, 25 cm × 0.25 cm I.D., 10 μm particle size (Laboratorní Přístroje, Prague, Czechoslovakia), a TZ-4200 double-line recorder (Laboratorní Přístroje) and a Varian-Techtron line recorder were used for the measurements. To evaluate the elution curves, a Model 3390A Hewlett-Packard reporting integrator was employed. Samples were injected through a 20-μl loop.

For the detection, an LC-UV photometric detector (Pye Unicam) and two electrochemical detectors were used. The voltammetric detector was of our own construction [35—37], operated in the wall-jet system with three electrodes and a polymeric carbon-paste working electrode (for the preparation of

the Alkapren paste see ref. 36). The polarographic detector was similar to that marketed by Princeton Applied Research, U.S.A., but with the eluate fed horizontally to the mercury-drop electrode. The electrochemical detectors were operated with prototype instruments from Laboratorní Přístroje, permitting d.c., damped d.c., sampled d.c. and differential pulse voltammetric measurements in a three-electrode system, with effective background current compensation. The potentials are related to saturated silver chloride electrode. During the measurement, the three detectors were connected in series — first the UV photometric detector, followed by the voltammetric and then the polarographic detector. The detectors were interconnected by pieces of stainless-steel capillary, 15 cm × 0.2 mm I.D.

Chemicals

The studied pyrimidine derivatives were obtained from Fluka, Buchs, Switzerland (6-azathymine), Calbiochem, Los Angeles, CA, U.S.A. (5-bromouracil, 6-azauracil, 5-iodouracil), Sigma, St. Louis, MO, U.S.A. (5-methylcytosine, 5,6-dihydrothymine), Lachema, Brno, Czechoslovakia (2,4,5-triamino-6-hydroxypyrimidine, 5-bromocytosine, cytosine, 5,6-dihydrothymine, uracil, 2-amino-4,6-dihydroxypyrimidine, 2,4-diamino-6-hydroxypyrimidine, 6-azacytosine), Hoffmann-La Roche, Basle, Switzerland (5-fluorouracil), Koch-Light, Colnbrook, Great Britain (thymine). 2-Mercapto-, 4-mercapto- and 2,4-dimercaptouracil, 2-mercapto-6-azathymine and 6-aminouracil were synthesized by Dr. Černohorský of the Department of Clinical Biochemistry (Prague, Czechoslovakia). 4,5,6-Triaminouracil, 6-amino-4-hydroxy-2-mercaptopyrimidine, 4,5-diamino-6-hydroxy-2-mercaptopyrimidine and 4,6-diamino-2-mercaptopyrimidine were provided by Dr. Žilka (ÚVVVR, Prague, Czechoslovakia).

Standard solutions of these substances in the mobile phase were always prepared immediately before the measurement.

The mobile phase consisted of 300 ml of 0.1 *M* citric acid and 160 ml of 0.1 *M* Na_2HPO_4 [38]; the pH was adjusted by appropriate addition of phosphoric acid and sodium hydroxide. It was deaerated by pre-purified nitrogen and degassed in vacuo. The mobile phase flow-rate was 1 ml/min.

All the measurements were carried out at laboratory temperature.

RESULTS AND DISCUSSION

Column separation

The reversed-phase system with a C_{18} alkyl-bonded silica column was selected on the basis of the good results reported in the literature (see Introduction). The phosphate—citrate mobile phase was chosen because of efficient separations attained with this system previously [38] and because citrate was reported as an optimal counter-ion in anion-exchange separation of nucleobases and nucleotides [11]. In this system, the capacity ratios were measured for the studied substances in dependence on the pH and are given in Table I and Fig. 1.

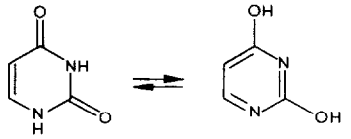
It is very difficult to explain the retention behaviour of pyrimidines on the basis of their structure, because of the simultaneous presence of acidic (—OH, —SH) and basic ($-NH_2$) ionizing groups and other substituents (—F, —Br, —I, etc.).

TABLE I

CAPACITY RATIOS OF VARIOUS PYRIMIDINE DERIVATIVES AND THE DETECTOR RESPONSE (AVERAGES OF THREE DETERMINATIONS)

Compound	Detector response		Capacity ratio			
	Voltammetric	Polarographic	pH 2.5	pH 3.5	pH 4.5	pH 6.0
2,4-Dihydroxypyrimidine (uracil)	—	—	0.99	1.13	1.25	1.26
2-Amino-4,6-dihydroxypyrimidine	+	—	0.29	0.65	0.60	0.34
4,5-Diamino-6-hydroxypyrimidine	+	—	0.22	0.58	1.15	1.24
2-Hydroxy-4-aminopyrimidine (cytosine)	—	—	0.43	0.66	0.79	1.04
2,4,5-Triamino-6-hydroxypyrimidine	+	—	0.13	0.28	0.30	0.65
2,4-Diamino-6-hydroxypyrimidine	+	—	0.88	1.72	1.50	1.39
2,4-Dihydroxy-6-aminopyrimidine	+	—	1.04	1.18	1.21	1.12
4,5,6-Triaminopyrimidine	+	—	0.87	1.58	1.43	2.12
2,4-Dihydroxy-5-methylpyrimidine (thymine)	—	—	3.36	3.73	4.35	4.60
5-Methylcytosine	—	—	1.02	1.78	2.28	3.49
5-Nitrouracil	—	+	1.12	1.50	1.73	1.75
2-Mercaptouracil	+	—	1.61	1.83	2.18	2.23
2,4-Dimercaptouracil	+	—	1.60	1.81	2.17	2.20
4-Mercaptouracil	+	—	2.73	3.12	3.72	3.85
2-Mercapto-4-hydroxy-6-aminopyrimidine	+	—	1.95	2.23	2.49	2.34
2-Mercapto-4,6-diaminopyrimidine	+	—	1.98	3.30	3.28	4.31
6-Azacytosine	—	+	0.58	0.70	0.73	0.75
6-Azauracil	—	+	0.75	0.88	0.95	0.95
6-Azathymine	—	+	2.30	2.77	3.17	3.65
2-Mercapto-6-azathymine	+	+	3.19	3.86	4.69	—
5-fluorouracil	—	—	1.01	1.03	1.37	1.37
5-Bromouracil	—	—	3.25	3.47	4.53	4.31
5-Iodouracil	—	—	5.25	4.94	7.49	—
5-Bromocytosine	+	—	1.90	3.90	5.36	—
5,6-Dihydrouracil	—	—	0.87	1.04	0.54	1.15
5,6-Dihydrothymine	—	—	3.23	3.05	3.74	3.72

Pyrimidines occur in two tautomeric forms, lactam and lactim; for example, for uracil,



lactam lactim

In reversed-phase systems, substances that are not ionized have high retention times; the k' value decreases with increasing ionization. The pyrimidines in which the lactam form predominates in aqueous solutions exhibit lower retention times. This phenomenon has been explained by self-association (vertical stacking) of the lactim form [24], leading to hydrophobization of the molecules and thus to longer retention times. Any substituent supporting the formation of the lactam form (e.g. —OH, —SH) causes a decrease in the capacity

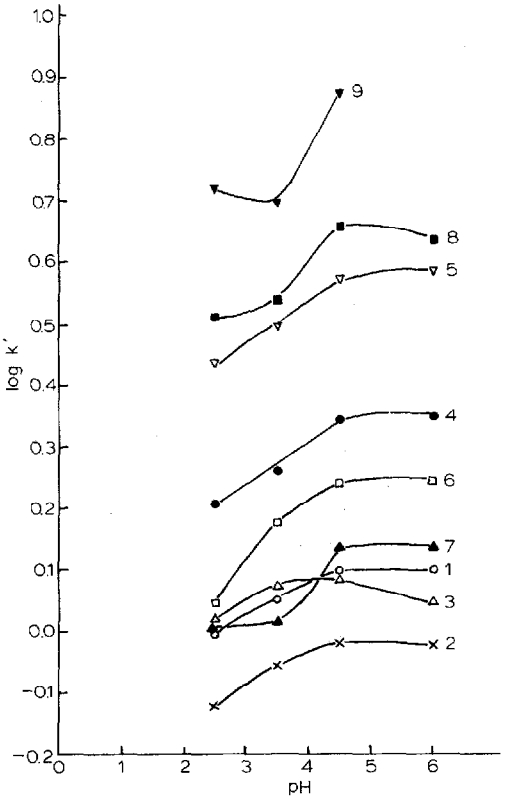


Fig. 1. Dependence of the logarithm of the capacity ratio (k') for some uracil derivatives on the pH of the mobile phase. 1 = Uracil, 2 = azauracil, 3 = 6-aminouracil, 4 = 2-mercaptouracil, 5 = 4-mercaptouracil, 6 = 5-nitrouracil, 7 = 5-fluorouracil, 8 = 5-bromouracil, 9 = 5-iodouracil.

ratio. The retention order of variously substituted pyrimidines increases in the series OH < H < NH_2 < CH_3, the greatest effect being exerted by the substituent in position 5. The presence of the methyl group in position 5 more than doubles the retention time, as it enhances the formation of the lactim form and thus also supports the stacking process (see k' for uracil and thymine, cytosine and 5-methylcytosine in Table I).

Fluorine, as an electron-withdrawing substituent in position 5, has little effect on the retention (see k' for 5-fluorouracil and uracil in Fig. 1). In the series of 5-fluoro-, 5-bromo- and 5-iodouracil, the retention order is inversely proportional to the electronegativity of the halogen atoms (Fig. 1). 6-Aza- and 5,6-hydrogeno- substitution lead to only small changes in the retention behaviour.

In agreement with Miller et al. [31], the optimum pH for the separation was found to be 3.5, since at higher pH values the elution curves exhibited progressively increasing tailing and sometimes even doubled peaks were obtained. The effect of pH on the capacity ratios is complicated, due to the simultaneous presence of acidic (—OH, —SH) and basic (—NH_2) groups and it cannot be directly correlated with the individual ionization constants; the effect of the

position of the substituent on the heterocycle is definitely more pronounced (cf. the great difference between k' values for 2-mercapto- and 4-mercapto-uracil and the elution order uracil (pK_a 9.38; 12.0) < 5-nitrouracil (pK_a 5.3; 11.7) < 2-mercaptouracil (pK_a 7.74; 12.7) in Fig. 1; the pK_a values were taken from Brown [39].

The effect of addition of dodecyl sulphate to the mobile phase on the separation was also tested. However, the retention times changed very little and increased peak tailing has been observed.

Detection

UV photometric detection at 254 nm is generally sufficiently sensitive for all pyrimidine derivatives. However, as follows from Table I, certain combinations of pyrimidine derivatives will not be resolved on the column and there may arise problems with identification of the individual components. Electrochemical detection depends on the presence of oxidizable or reducible groupings in the solute molecule and thus is selective for certain derivatives. The half-wave potentials of the voltammetric waves vary widely (e.g. + 0.45 V for 2,4,5-triamino-6-hydroxypyrimidine, + 1.15 V for 2,4-diamino-6-hydroxypyrimidine and + 1.2 V for 2-amino-4,6-dihydroxypyrimidine [40]) and therefore the selectivity of the detection can further be varied by varying the potential applied to the working electrode. To test the possibilities of electrochemical detection, the potentials of the working electrodes were set at limiting values, given by the mobile phase composition, i.e. + 1.4 V for the carbon-paste voltammetric wall-jet detector and − 1.0 V for the mercury-drop electrode polarographic detector.

The results can be seen in Table I: the voltammetric detector responds to substances with oxidizable groups, i.e. amino and mercapto, and the polarographic detector to reducible groups, i.e. nitro and aza. It is important that the signal magnitude is proportional to the number of the electroactive groups (e.g. the detection limits are 5, 10 and 25 ng for tri-, di- and monoamino derivatives) due to the varying number of electrons exchanged per solute molecule.

The detection limits for typical solutes are given in Table II. It can be seen that, except for 4,5,6-triaminopyrimidine, the sensitivity of electrochemical detection is not greater than that of UV photometric detection. The detection limit of the polarographic detector is even considerably higher than that of the UV photometric detector. However, the combination of UV photometric detector with the electrochemical detectors can substantially improve the possibilities of resolving and identifying the components of the mixture, especially in biological fluids, in which most ballast substances absorbing in the UV region will not yield an electrochemical response. In these experiments, the detectors were connected in the series, UV—voltammetric—polarographic, by the shortest possible steel capillaries with an internal diameter of 0.2 mm. As the internal volume of the UV detector cell is 5 μl and that of the voltammetric detector ca. 1 μl, the elution curves are not appreciably broadened even in the polarographic detector recording. The voltammetric detector is partially destructive; however, the residence time of the substance inside the cell is so short that the degree of electrochemical conversion is very low. For this reason, the oxidation products generated in the voltammetric detector that might be re-reduced in the polaro-

graphic detector, thus interfering with its performance, were not actually detected. The precision of the determination with the electrochemical detectors is satisfactory for quantitative analysis (see Table II).

There is another possibility of electrochemically detecting substances eluted from a chromatographic column. If a mercury electrode is polarized at a potential corresponding to the dissolution of mercury, then diffusion-controlled anodic waves are formed in the presence of substances that form stable complexes or precipitates with mercury ions (see e.g. ref. 41). Most organic substances separated by HPLC would form complexes with mercury ions; there-

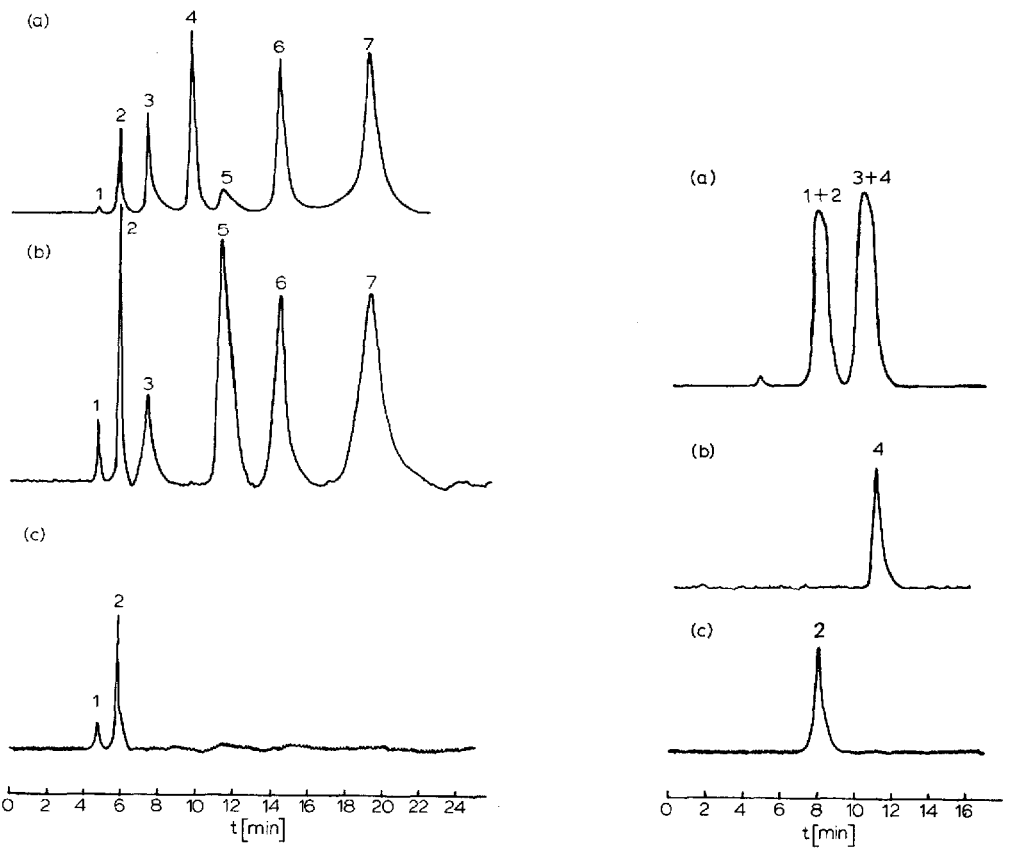


Fig. 2. Separation of several pyrimidine derivatives. (a) UV photometric detection at 254 nm (sensitivity 0.32 absorbance units/scale). (b) Voltammetric detection, +1.4 V (sensitivity 0.2 μA/scale). (c) Voltammetric detection, +0.8 V (sensitivity 0.2 μA/scale). Mobile phase pH, 3.5; for other conditions see the experimental part. 1 = Hold-up time; 2 = 2,4,5-triamino-6-hydroxypyrimidine, 1.12 μg, 3 = 2-amino-4,6-dihydroxypyrimidine, 0.88 μg, 4 = uracil, 0.56 μg, 5 = 4,5,6-triaminopyrimidine, 0.64 μg, 6 = 6-amino-4-hydroxy-2-mercaptopyrimidine, 0.40 μg, 7 = 4-mercaptouracil, 1.50 μg.

Fig. 3. Chromatograms of cytosine (1) (0.65 μg), 6-azacytosine (2) (1.2 μg), uracil (3) (0.56 μg), and 6-aminouracil (4) (0.60 μg). (a) UV photometric detection at 254 nm (sensitivity 0.32 absorbance units/scale). (b) Voltammetric detection, + 1.4 V (sensitivity 0.2 μA/scale). (c) Polarographic detection, −1.0 V (sensitivity 0.05 μA/scale). Mobile phase pH, 3.5; for the other conditions see the experimental part.

TABLE II

TYPICAL DETECTION LIMITS AND REPRODUCIBILITY OF MEASUREMENT

Detection limits were determined as three times the peak-to-peak noise values.

Substance	Detection limit (ng)			Reproducibility of measurement (relative S.D., %)				
	UV	Voltammetric	Polarographic	UV		Voltammetric		Polarographic
				Height	Area	Height	Area	Height
2-Amino-4,6-dihydroxypyrimidine	2	25	—					
2,4-Diamino-6-hydroxypyrimidine	2	10	—					
2,4,5-Triamino-6-hydroxypyrimidine	2	5	—	1.53*	1.24*	5.18*	5.92*	—
4,5,6-Triaminopyrimidine	20	7	—					
2-Mercaptouracil	10	50	—					
5-Nitrouracil	2	—	1000	1.72**	—	—	—	2.25**
6-Azauracil	2	—	800					
6-Azacytosine	5	—	400					
2-mercapto-6-azathymine	10	40	800					

*1.32 μg, seven measurements.
**2.8 μg, four measurements.

fore, this possibility was tested, setting the mercury electrode potential at + 0.5 V. There was a response to the substances eluted from the column; however, the sensitivity was too low. The obvious reason for the low sensitivity was the fact that the substance to be detected was carried away from the electrode before the chemical reaction of complexation or precipitation could proceed to a greater extent. Therefore, it seems that the use of chemical reactions with mercury ions that have a substantial importance, e.g. in stripping analysis [42], will be unimportant in flow systems because of too slow kinetics of the overall process.

The advantages following from combination of the UV photometric detection with voltammetric detection at various electrode potentials and further with polarographic detection are illustrated in Figs. 2 and 3. This selective detection will be utilized in the determination of active components based on the substances studied in pharmaceuticals and in some biological samples when following their metabolism. The selectivity of detection and improved separation will be especially advantageous in analyses of biological samples with complicated matrices.

CONCLUSIONS

The experiments carried out have shown that a reversed-phase HPLC separation of pyrimidine derivatives can be used even for very complex mixtures. The resolution and identification of the components is facilitated by combined UV–voltammetric–polarographic detection.

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