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Reversed-phase liquid chromatography using monoalkylammonium compounds in the mobile phase

Effects of monoalkylammonium chain length on the efficiency, selectivity and separation of xanthine and uric acid derivatives

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ABSTRACT

A systematic analysis of the influence of several chromatographic parameters on the adsorption of monoalkylammonium modifiers on a chemically bonded phase in reversed-phase high performance liquid chromatography is presented. The most important variables are the alkyl chain length, the concentration of the monoalkylammonium modifiers and the acetonitrile content in the mobile phase. The results can be applied to the separation and structure determination of xanthine and uric acid derivatives and to the study of retention mechanisms.

INTRODUCTION

The chromatographic performance on chemically bonded-phase high-performance liquid chromatographic (HPLC) columns of solutes containing amine functional groups, such as xanthine and uric acid derivatives, is often poor. The chromatography of these derivatives can be improved by adding a modifying agent to the mobile phase [1]. Optimization of the retention and selectivity of these derivatives has been carried out by an appropriate combination of the composition and pH of the mobile phase and by adding a suitable alkylamine modifier, changing the chromatographic mode [reversed-phase (RP) HPLC] into reversed-phase ion-pair partition [2].

The use of octadecylsilyl (ODS) phases dynamically modified by adding ion-pairing reagents, such as dioctylammonium [3], dimethyloctylammonium [4], hexylammonium [5,6], dibutylammonium [7], tetrabutylammonium [8,9], cetyltrimethylammonium [10–12] and decylammonium ion [13,14], to the eluent leads to a better chromatographic behaviour and helps to resolve problems associated with retention, column efficiency and peak symmetry [15,16]. The theoretical dependence of the capacity factors (k') on the concentration of the ion-pair reagent, on the pH values of the mobile phase and on the pK_a values of the solutes has been discussed by several workers [11,17–21]. A practical equation based on the electrostatic model of reversed-phase ion-pair chromatography (RP-IPC) describes the relationship between the capacity factor of ionic

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solutes and the eluent concentration of the modifier [9,22–24]. However, no exhaustive study on the conditions of use of monoalkylammonium ions has yet been reported [3,25–27].

The purpose of this study was to investigate the adsorption of different monoalkylammonium ions on the ODS surface and to determine their influence on the chromatographic behaviour of uric acid and xanthine derivatives.

EXPERIMENTAL

Apparatus

Chromatography was performed on a Varian 5000 instrument equipped with a stainless-steel Ultrasphere IP (5 μm) column (15 cm × 0.4 cm I.D.) (Beckman Instruments, Berkeley, CA, USA). UV detection was performed with a Spectroflow 773 spectrophotometer (Kratos Analytical Instruments, Westwood, NJ, USA) set at 280 nm. A CR-3A peak integrator (Shimadzu, Kyoto, Japan) was used and automatic injections of 20 μl were performed with a Model 231 injector (Gilson Medical Electronics, Middleton, WI, USA). The flow-rate was 2 ml/min. For the breakthrough curves, a Shodex RI SE-11 differential refractometer (Showa Denko, Tokyo, Japan) was used.

Chemicals

Doubly distilled water was used throughout.

Theophylline (1,3-dimethylxanthine, 1,3-DMX), dyphylline and caffeine (1,3,7-trimethylxanthine, 1,3,7-TMX) were obtained from Sigma (St. Louis, MO, USA), 3-methylxanthine (3-MX), 7-methylxanthine (7-MX), 1-methylxanthine (1-MX), 1,7-dimethylxanthine (1,7-DMX), 1-methyluric acid (1-MU), 3-methyluric acid (3-MU), 7-methyluric acid (7-MU), 9-methyluric acid (9-MU), 1,7-dimethyluric acid (1,7-DMU), 3,7-dimethyluric acid (3,7-DMU), 1,3-dimethyluric acid (1,3-DMU), 1,9-dimethyluric acid (1,9-DMU), methylamine · HCl, ethylamine · HCl, *n*-propylamine, *n*-butylamine, *n*-pentylamine, *n*-hexylamine, *n*-heptylamine, *n*-octylamine, *n*-nonylamine, *n*-decylamine, *n*-undecylamine, *n*-dodecylamine, *n*-tetradecylamine, *n*-hexadecylamine and *n*-octadecylamine from Fluka (Buchs, Switzerland) and acetonitrile from Merck (Darmstadt, Germany).

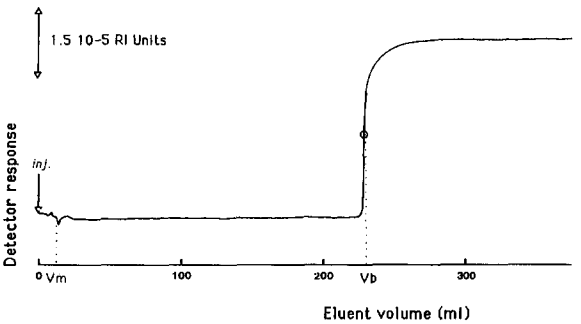

Fig. 1. Breakthrough curve of decylammonium ion using a differential refractometer (range ×8). Eluent, acetonitrile–85 m*M* acetate buffer (pH 4.0) (2:98, v/v); decylammonium ion, 0.75 m*M*; temperature, 25°C; flow-rate, 2 ml min^{-1}. V_b is the breakthrough volume.

Column loading and equilibration

The amounts of *n*-alkylammonium ions adsorbed by the stationary phase from the standard eluent were determined by the breakthrough method [19, 28–30] (Fig. 1). Before breakthrough volume measurement, the HPLC columns were washed successively with acetonitrile and the degassed mobile phase (standard eluent) prepared with 85 m*M* sodium acetate buffer (pH 4.0)–acetonitrile (98:2, v/v).

The standard eluent containing 0.75 m*M* *n*-alkylamine was used for the adsorption diagrams and the standard eluent containing *n*-decylamine (0.38–3.75 m*M*) was used for the isotherm adsorption.

The breakthrough volume, V_b, was measured from the moment of changing the standard eluent to the mid-point of the eluted front. The *n*-alkylammonium ion concentration in the stationary phase (C_{st}, μmol g^{-1}) was calculated using the equation

$$C_{st} = \frac{(V_b - V_0)C_m}{W_{st}} = \frac{Q_{ads}}{W_{st}}$$

where C_m (mmol l^{-1}) is the concentration of pairing ions in the mobile phase, V_b is the breakthrough volume, V_0 is the void volume of the chromatographic system, W_{st} (g^{-1}) is the packing within the column and Q_{ads} is the amount of pairing ion adsorbed in the stationary phase [19,25].

RESULTS AND DISCUSSION

Effect of alkyl chain length on n-alkylamine adsorption

After running a mobile phase containing 0.75 m*M* of the alkylammonium ion, the amounts of alkylamines containing 1–16 carbon atoms adsorbed were determined. The structures of the alkylamines used were $CH_3(CH_2)_{n-1}NH_2$ ($1 \leqslant n \leqslant 16$). The scope of the study was determined by the solubility characteristics of the alkylammonium ions in the mobile phase. Amines containing 18 carbon atoms or more could not be used as they were not soluble under our experimental conditions.

Several workers have observed linear relationships between log C_{st} and the number of carbon atoms in the alkyl chain of the modifier for homologous series [25]. Alkylammonium ions adsorption can be expressed by

$$\log C_{st} = an + b$$

where n is the number of carbon atoms in the alkyl chain of the modifier and a and b are constants for a given set of experiments.

Fig. 2 shows the exponential variation of alkylammonium ion concentration in the stationary phase (C_{st}) *versus* n. Primary monoalkylamines containing 1–4 carbon atoms are not fixed on the column; the corresponding ammonium cations are too hydrophilic. For alkylammonium ions containing 5–10 carbon atoms, the amount of alkylammonium ions adsorbed in the stationary phase (Q_{ads}) increases with increasing length of the alkyl chain, and the fixation is linear. If we use the adsorption isotherm referred to by Ståhlberg [23], we deduce from the slope in Fig. 2 that the increase in adsorption energy per CH_2 group is 1.9 kJ mol^{-1} [31]. This value is lower than that obtained for the adsorption of this kind of ion between water and oil (2.88–2.97 kJ mol^{-1} [32,33]) and the reason is probably the presence of acetonitrile in the mobile phase [34].

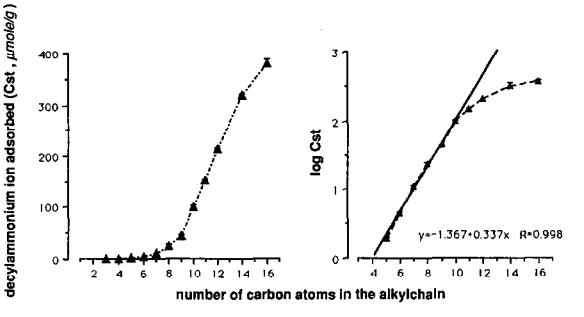

Fig. 2. Adsorption of alkylammonium ion by a 15-cm Ultrasphere IP (5 µm) column as a function of the number of carbon atoms in the alkyl chain in the mobile phase [acetonitrile–85 m*M* acetate buffer (pH 4.0) (2:98, v/v); alkylammonium ion, 0.75 m*M*]. Temperature, 25°C; flow-rate, 2 ml min^{-1}.

However, when $n > 10$ the variations are irregular. In this instance, the lower efficacy of the fixation can be explained by the electrostatic theory of ion-pair chromatography [23,24]. A surface potential exists whenever there is an excess of charged species of one type of sign over species of the opposite sign on the surface. The non-linearity of the adsorption isotherm of n-alkylammonium ions is due in turn to repulsion from the electrical double layer created and to the decrease in available surface area [23,35].

Table I shows the percentage occupation of the octadecyl site; the stationary phase used (Ultrasphere IP) contains 11.7% of carbon, which is equivalent to 541 µmol of octadecyl chains per gram of stationary phase. Under the experimental conditions chosen ($5 < n < 10$), the percentage occupation of the octadecyl sites is limited (<20%). Then, neutral derivatives undergo a dual mechanism of retention owing to the hydrophobic interactions

TABLE I

OCCUPATION OF THE STATIONARY PHASE FOR A CONCENTRATION OF 0.75 m*M* OF ALKYLAMMONIUM ION IN THE MOBILE PHASE

Alkyl chain length, n	Occupation of octadecyl sites (%)
6	0.8
7	2
8	4
9	8
10	18.5
11	28
12	39
14	59
16	71.2

with the stationary and mobile phases, while negatively charged species are retained by an ion-pair mechanism [1,7,22,23,34–36].

Under our experimental conditions (pH 4.0, 2% acetonitrile), the choice of the decylammonium ion permits the separation of the puric derivatives. The decylammonium ion is then characterized by a regular fixation with a percentage occupation of the octadecyl sites that is sufficiently low to determine an appropriate modulation for the retention of derivatives. This modifier adsorption decreases the k' of uncharged species and increases that of anionic species.

Adsorption isotherm; effect of decylammonium concentration in mobile phase

Ståhlberg [23] developed an electrostatic retention model for RP-IPC. The phenomenon of adsorption at the bonded phase–eluent interface involves an electrical double layer, creating an electrostatic surface potential [25,33,34,37]. This adsorption of the modifier can be described by a Langmuir equilibrium in which allowance is made for the electrical contributions to the adsorption energy, and also by a Freundlich isotherm [34]. In a number of instances (cetyltrimethylammonium ion [37], tetralkylammonium ion [25], alkylbenzyldimethylammonium ion [38] and hexylammonium ion [6]), adsorption of long-chain amines was well fitted by the following empirical relationship:

$$C_{st} = \alpha(C_m)\beta \quad \text{or} \quad \log C_{st} = \alpha' + \beta \log C_m$$

where α, α' and β are constants for a given set of experiments.

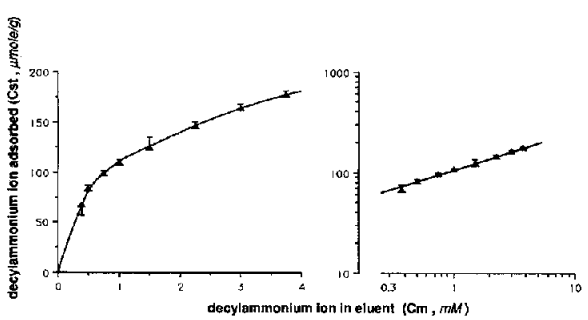

Fig. 3. Adsorption isotherms of decylammonium ion on a 15-cm Ultrasphere IP (5 μm) column from standard eluent [acetonitrile–85 mM acetate buffer (pH 4.0) 2:98, v/v)]. Temperature, 25°C; flow-rate 2 ml min^{-1}.

Fig. 3 shows that the adsorption isotherm of decylammonium ion from the standard eluent on to the ODS phase is fitted by the Freundlich equation over an aqueous concentration range of 0.38–3.75 mM at pH 4.0. Under these conditions the variation of log C_{st} is linear and increases with increasing concentration of decylammonium ions in the mobile phase. The experimental isotherm is described by the equation

$$\log C_{st} = 2.026 + 0.398 \log C_m \quad (R = 0.985)$$

Influence of acetonitrile content in mobile phase

Under our experimental conditions with slightly soluble alkylammonium ions (decylamine concentration in the mobile phase less than 1 mM) and an acetonitrile content varying from 0.25 to 4%, it was possible to establish a regular fixation without losing column efficiency.

It is known that retention values in RP-IPC are dependent on the concentration of organic solvent in the mobile phase [39 42]. The dependence of retention values on the concentration of the ion-pair reagent has been discussed by several workers [11,17–21,25,40–42]. Acetonitrile decreases the surface concentration of the adsorbed ion-pairing reagent [41]. Fig. 4 shows that the fixation of decylammonium ions decreases when the acetonitrile content increases. This adsorption can be expressed by the equation [25,41]

$$\log[C^+]_{st} = X - Y[\text{ACN}]$$

where X and Y are constants for a given set of experiments and [ACN] is the acetonitrile content in the mobile phase.

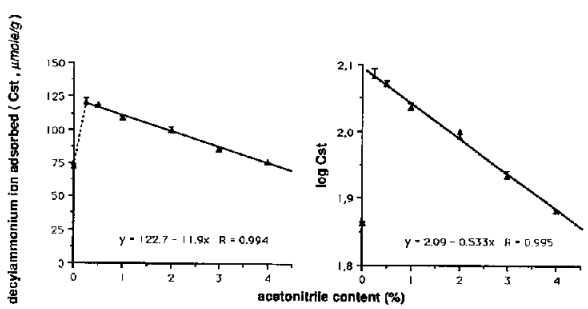

Fig. 4. Adsorption of decylammonium ion by a 15-cm Ultrasphere IP (5 μm) column as a function of acetonitrile content in the mobile phase [acetonitrile–85 mM acetate buffer (pH 4.0); alkylammonium ion, 0.75 mM]. Temperature, 25°C; flow-rate 2 ml min^{-1}.

Regarding the chromatographic behaviour of xanthine and uric acid derivatives, for a volume fraction of acetonitrile higher than 4%, it has been observed that the elution is very rapid and that the theophylline metabolites are not separated [14]. At concentrations lower that 0.25%, the mechanism of separation in RP-IPC is altered.

Under our experimental conditions, the presence of acetonitrile in the mobile phase reduces the free energy of the modifier adsorption usually found for this kind of ion [9,23,31–34,41,42]. The addition of acetonitrile modifies the dielectric constant, the viscosity and the superficial tension at the bonded phase–eluent interface. The substantial lowering of the superficial tensions, even at the supposedly low percentages of acetonitrile, favours the accessibility of the alkylammonium chains to the octadecylsiloxane sites [39].

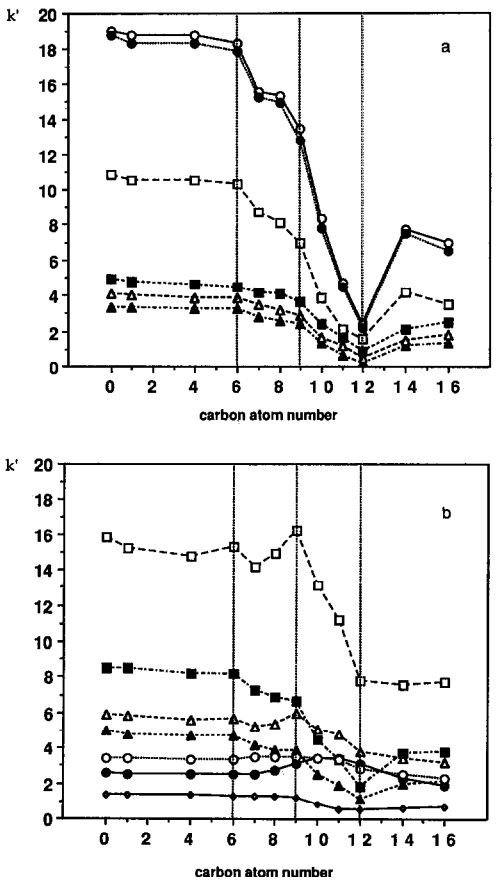

Fig. 5. Effect of alkyl chain length on the retention of (a) xanthine derivatives and (b) uric acid derivatives. (a) ○ = 1,3-DMX; ● = 1,7-DMX; □ = 3,7-DMX; ▲ = 7-MX; △ = 3-MX; ■ = 1-MX. (b) ▲ = 3,7-DMU; △ = 1,9-DMU; ■ = 1,3-DMU; □ = 1,7-DMU; ◇ = 3-MU; ● = 7-MU; ○ = 1-MU.

Chromatographic results and retention mechanisms

Alkylammonium ions have been applied to the separation of puric acid derivatives. Decylamine, in particular, has been used to separate theopylline metabolites [14]. Solute retention has been reported to vary with the chain length of the pairing ion [18,25,43,44]. The variations of the capacity factors (k') of uric acid and xanthine derivatives with the size of the alkyl chains are shown in Fig. 5. These variations divide the alkylammonium ions into four groups.

Group 1 is composed of alkylammonium ions which have an alkyl chain containing six carbon atoms or less. These ions reduce by less than 6% the k' values, expressed as relative values compared with the k' values determined in a mobile phase devoid of alkylamine. Such variations are negligible, being within the limits of experimental errors. This absence of variation confirms that alkylammonium ions with short chains have no effect on separation mechanisms. These constituents are entirely in the mobile phase, and in our experiments they had no effect on apolar, polar or ionic solutes; in particular, no ion pairing was observed.

Group 2 consists of alkylammonium ions which have an alkyl chain containing six to nine carbon atoms; these divide the derivatives of xanthine and

TABLE II

pK_a VALUES OF PURINE DERIVATIVES [45,46]

Derivative	pK_a	Derivative	pK_a
7,9-DMU	5.05	9-MX	6.12
9-MU	5.10	1,3-DMU	6.22
1,9-DMU	5.18	1-MX	7.90
1,7,9-TMU	5.28	7-MX	8.42
7-MU	5.45	3-MX	8.45
1-MU	5.48	1,7-DMX	8.65
1,7-DMU	5.93	1,3-DMX	8.68
1,9-DMX	5.99	3,9-DMU	8.91
1,3,7-TMU	6.01	1,3,9-TMU	9.39
3-MU	6.02	3,7,9-TMU	9.42
3,7-DMU	6.04	3,7-DMX	10.00
		3,9-DMX	10.14

uric acid into two categories: derivatives whose k' values decrease from 17% to 34% are the derivatives of xanthic and dimethyluric acids with methyl substituents in position 3 [N(3)–CH_3] and with a pK_a above 6 (Table II, Figs. 6 and 7); and derivatives whose k' values increase from 5% to 26% are the derivatives of uric acid with a pK_a below 6, corresponding to the N(3)–H-related acidic character expressed by C(2)–O^- (Table II, Figs. 6 and 7).

In group 2, when significant amounts of alkylammonium ions are adsorbed on the ODS surface, compounds without acidic character (non-ionic solutes such as xanthine derivatives and uric derivatives with $pK_a > 6$) are chromatographed according to a reversed-phase partition mechanism. The acidic compounds (7-MU, 1-MU and 1,9-DMU with $pK_a < 6$) are separated by reversed-phase ion-pair partition or by an intermediate mechanism.

Group 3 ($10 \leqslant n \leqslant 12$) is characterized by a decrease in all k' values which is minor (8–25%) for uric acid derivatives with $pK_a < 6$, despite an increase in cationic charge density in the stationary phase. On the other hand, the marked fall (>40%) observed with all the other derivatives can be attributed to the overcrowding resulting from an increase in the amount of *n*-alkylammonium ions adsorbed by the stationary phase, which limits the "reversed-phase partition phenomena". The adsorption of the pairing ion causes a decrease in the available hydrophobic surface area [43,44].

In group 4, which includes the very long-chain alkylammoniums ($n > 12$), a major increase in k' values was observed for xanthine derivatives and uncharged uric acid solutes (relative variation above 172%), whereas the k' values of anionic uric acid derivatives (7-MU, 1-MU and 1,9-DMU) decreased down to C_{16}. With xanthic derivatives and uncharged derivatives 3-MU, 1,3-DMU and 3,7-DMU, the effect of reduced accessibility to apolar sites was maximum for $n = 12$. For anionic uric derivatives ($pK_a < 6$), a decrease in k' beginning with $n = 11$ was confirmed for group 4 with $14 \leqslant n \leqslant 16$, whereas in this group the uncharged xanthic and uric solutes had increased or erratic retentions.

The results obtained orient the choice of the modifier towards a 9–10-carbon chain length, at the junction of groups 2 and 3 as defined above.

General comments on the structure of the xanthine and uric acid derivatives

The general structure and anionic forms of xanthine derivatives are shown in Fig. 6 and those of uric acid derivatives in Fig. 7. The similarity of behaviour in each class, demonstrated in Fig. 5, makes it possible to compare the behaviours of various solutes.

The 7-MU and 1-MU solutes (anionic uric derivatives) with $pK_a < 6$) have a particular behaviour with k' values that are maximum for $n = 10$ and minimum for $n = 16$ (Fig. 5b). The increase in retention resulting from ion pairing is significant with 7-MU and smaller with 1-MU. This result can be ascribed to the structural behaviour. With both solutes, the anionic form responsible for the pairing is C(2)–O^- (Fig. 7) [46]. The regular ion pairing observed with 7-MU is limited with 1-MU by steric hindrance due to the methyl group in position 1, which limits the accessibility of the solute to the cationic sites of the stationary phase.

The chromatographic behaviour of the 3-MU solute is identical with that of the monomethylxanthic derivatives and is characterized by a decrease in k' values for $n = 12$ (Fig. 5b). At pH 4.0, this solute ($pK_a = 6.02$) is in the uncharged lactam form (2,4,6-trioxo), with a pyrimidinic structure that is closer to the monomethylxanthines at this pH (Fig. 7). With 3-MU, the first ionic dissociation is in the N(7)–H position corresponding to the C(8)–O^- structure showing an electronic charge distribution that is different from that of the 7-MU and 1-MU derivatives [first ionic dissociation in the N(3)–H position corresponding to the C(2)–O^- structure] [46,47]. At pH 4.0, the ionic dissociation of 3-MU is negligible, but apart from its peculiar polarizability the maximum conjugation of hydration sites explains why this solute has a greater polarity than the other monomethyluric derivatives. The order of elution is 3-MU > 7-MU > 1-MU.

These 1-MX, 3-MX and 7-MX monomethylxanthines (Fig. 5) are characterized by a V-shaped variation of k' values, falling for $n = 12$, then rising until $n = 16$. These solutes with pK_a around 8 (Table II) are non-ionic at pH 4.0 and highly sensitive to the decrease in the number of apolar sites of the charged ODS. The order of elution is 7-MX > 3-MX > 1-MX. This results in solvation interactions (less with 1-MX, which limits the hydration site conjuga-

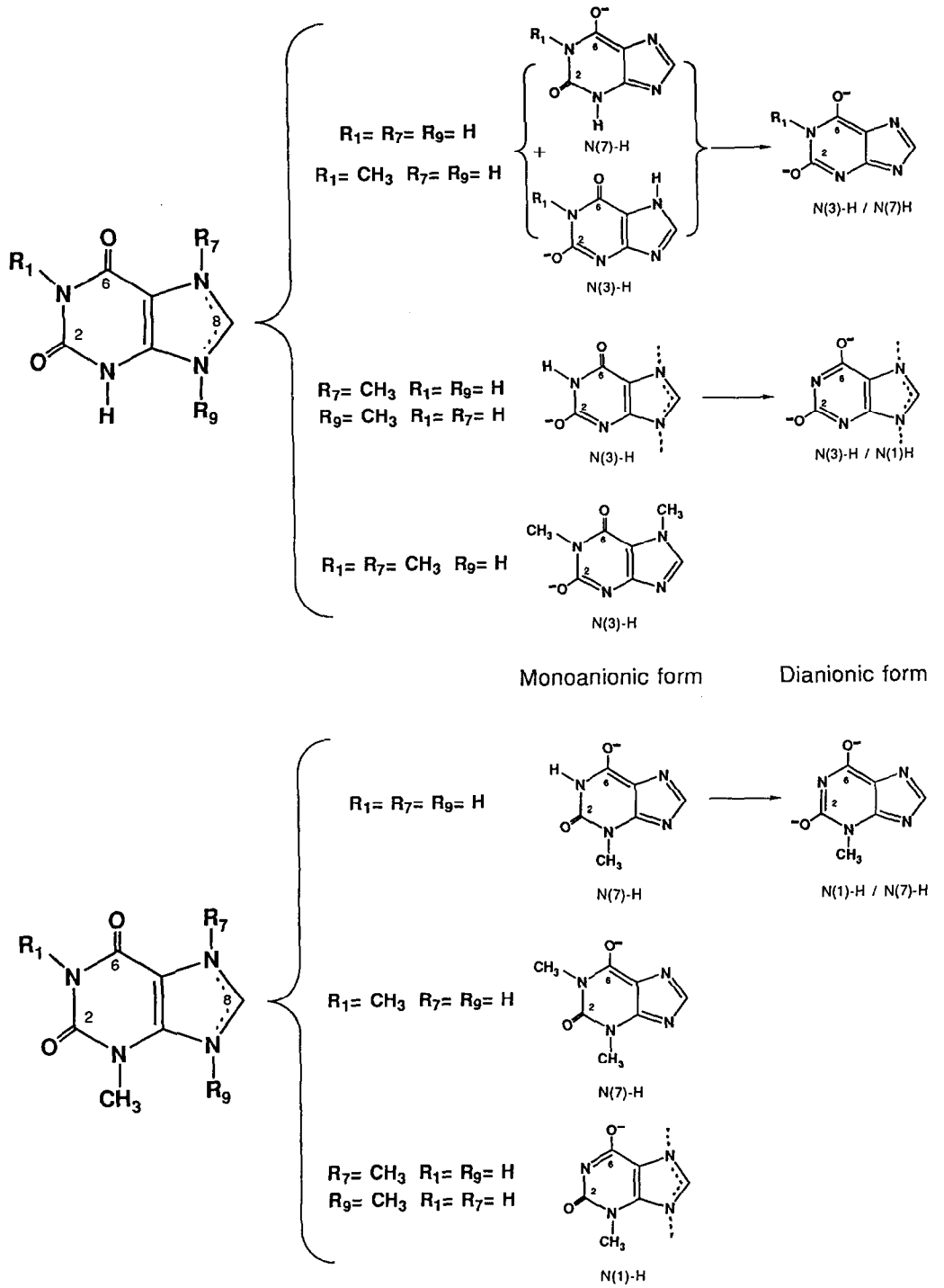

Fig. 6. General structures and ionic forms of xanthine derivatives.

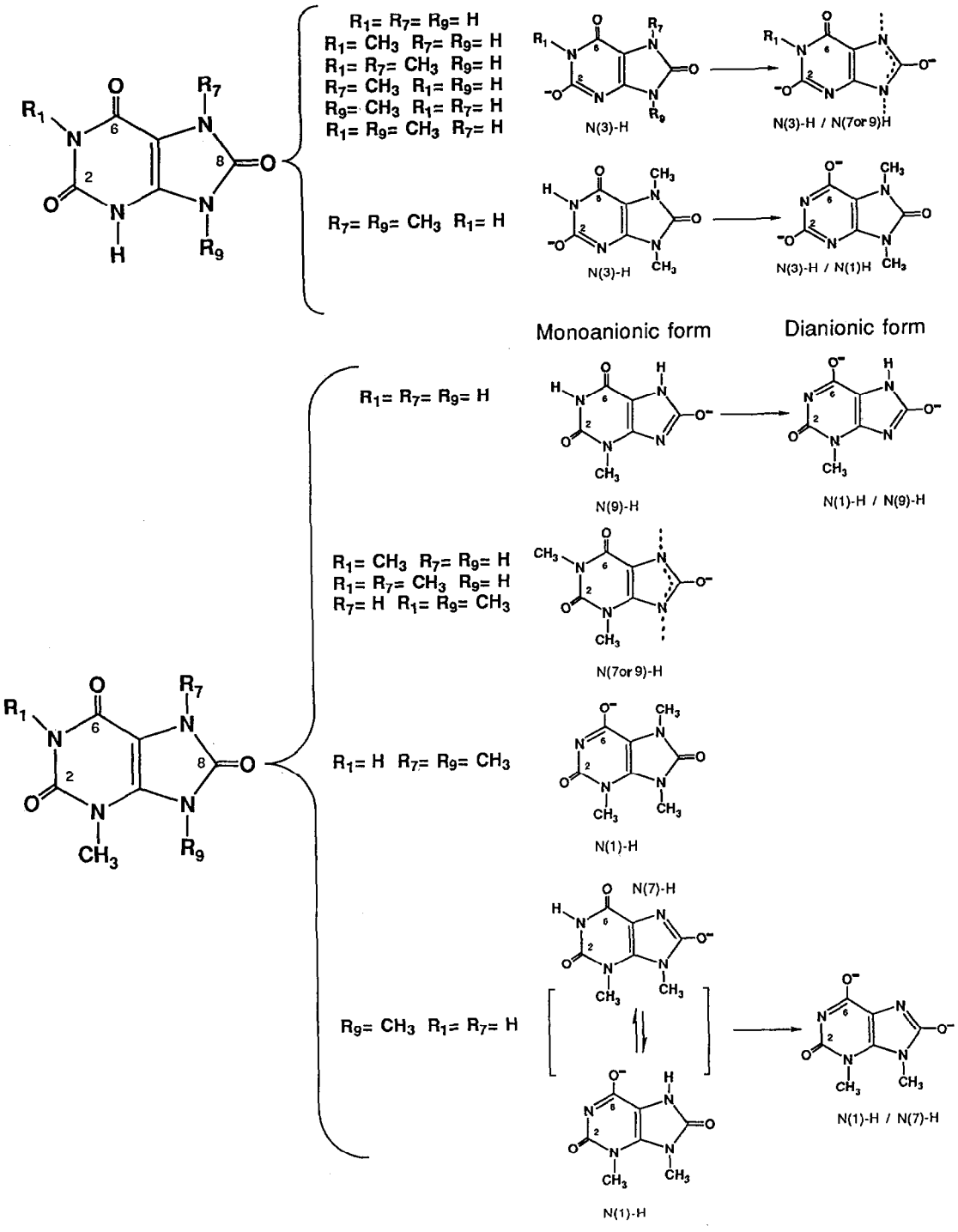

Fig. 7. General structures and anionic forms of mono- and dimethyluric acid derivatives.

tion) and in differences in cyclic anisotropy: a methyl substitution on N(7) of the xanthine derivatives ensures an increase in charge on C(8), which is greater than that resulting from methylation on N(3) or N(1) [N(3)-CH_3 > N(1)-CH_3 effect) [47,48].

The chromatographic behaviour of dimethylxanthines is similar to that of monomethylxanthines (decrease in k' values for $n = 12$) (Fig. 5a), with a more pronounced and hydrophobic character for 1,7-DMX and 1,3-DMX.

Dimethyluric derivatives with $pK_a > 6$ [those with a substitution on N(3) such as 3,7-DMU and 1,3-DMU] show k' variations that are similar to those of xanthine derivatives, thus confirming their low polar character under the experimental conditions (Fig. 5a and b).

The most acidic dimethyluric derivatives [with substitution on the N(9) or N(7) position, such as 1,9-DMU and 1,7-DMU (Fig. 5b], have an intermediate behaviour without any significant change in k' values until $n = 10$, then a fall for $n = 12$ and a constant value for $n > 12$.

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