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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF XANTHINES

EFFECTS OF N-METHYL AND C-8 HYDROXYL SUBSTITUTION ON THE RETENTION TIMES

T.B. VREE*, L RIEMENS and P.M. KOOPMAN-KIMENAI

Department of Clinical Pharmacy, Sint Radboud Hospital, University of Nijmegen, Nijmegen (The Netherlands)

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SUMMARY

The capacity factors of 26 xanthine derivatives were measured by reversed-phase high-performance liquid chromatography. N-Methyl substitution increased the capacity factor and the related lipid solubility. The descending order of the increase in capacity factor by the N-methyl group is: N-1 methyl > N-3 methyl > N-7 methyl > N-9 methyl. C-8 hydroxylation reduces the capacity factor in the xanthines. The reduction factor is 3.34 in xanthines with N-3 methyl substitution, 2.41 in xanthines with N-1 and/or N-7 methyl substitution and 1.68 in xanthines with N-9 methyl substitution.

INTRODUCTION

The xanthines caffeine (1,3,7-trimethylxanthine; 1,3,7-MX) and theobromine (3,7-dimethylxanthine; 3,7-MX) are constituents of tea and coffee, and cocoa and chocolate, respectively. They are thus food constituents of humans. Theophylline (1,3-dimethylxanthine; 1,3-MX) is a mild diuretic agent, a moderately myocardial and central nervous stimulant and a powerful bronchodilator. The trimethylxanthine caffeine is extensively metabolized into predominantly the dimethylxanthine paraxanthine (1,7-dimethylxanthine; 1,7-MX) and to a minor extent to theophylline and theobromine. Each of the dimethylxanthines is metabolized into monomethylxanthines and hydroxylated at the C-8 position. Patients on theophylline medication for obstructive airway diseases and consuming caffeine-containing beverages excrete the whole spectrum of metabolites in the urine. High-performance liquid chromatographic (HPLC) methods that enable the separation and identification of all possible metabolites are required for caffeine and theophylline analysis [1–7]. The chromatographic behaviour of the

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xanthines is the result of the intrinsic and partial contribution of each substituent of the xanthine skeleton to the interaction with the mobile phase, the solute and the stationary phase.

The aim of this study is to investigate the intrinsic contributions of the N-methyl and C-8 hydroxyl substituents to the chromatographic process.

EXPERIMENTAL

Chromatography

A Spectra Physics SP 8810 pump (Spectra Physics, Eindhoven, The Netherlands) was connected to a Kratos Spectroflow 770 detector (Kratos, Rotterdam, The Netherlands) and a BD7 recorder (Kipp & Zonen, Delft, The Netherlands). The stainless-steel column (250 mm×4.6 mm I.D.) was prepacked with Spherisorb 5-ODS, particle size 5 μm (Chrompack, Middelburg, The Netherlands). A stainless-steel column (75 mm×2.1 mm I.D.) packed with pellicular reversed phase (Chrompack) was used as a guard column.

The mobile phase (pH 5.30), water–acetonitrile–0.1 *M* acetic acid (81.5:6.0:12.5, v/v/v) with 0.87 g of sodium acetate trihydrate (Merck, Darmstadt, F.R.G.), was filtered through a Millipore 0.5-μm filter, type FH (Milford, MA, U.S.A.) and degassed with helium before use. The flow-rate was 1.5 ml/min, and chromatography was performed at room temperature with detection at 280 nm.

Retention times were measured at room temperature, and the column dead-time, t_0, was determined using methanol as the non-retained compound. The capacity factor, k', is defined as: $k' = (t_R - t_0)/t_0$, where t_R is the retention time of the solute.

Solutes

The xanthines were obtained from Fluka (Buchs, Switzerland; Hicol, Rotterdam, The Netherlands). The structures of four xanthines are shown in Fig. 1. Stock solutions of 500 μg/ml were prepared in methanol–0.1 *M* borax buffer (OPG, Utrecht, The Netherlands) (40:60, v/v) and were stable when stored at 4°C.

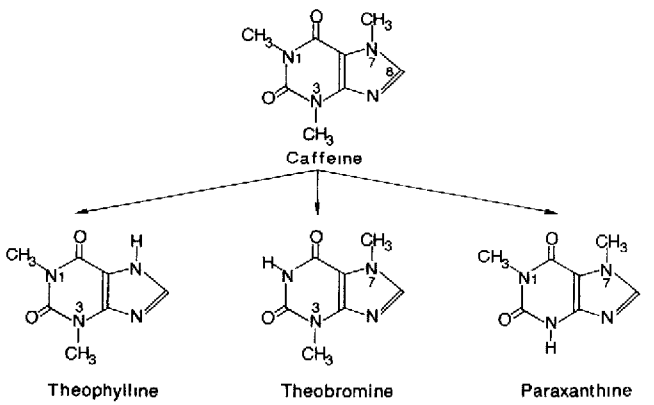

Fig. 1. Structures of the xanthines caffeine, paraxanthine, theobromine and theophylline.

TABLE I

CAPACITY FACTORS OF A SERIES OF XANTHINES

Compound	k'
Uric acid, U	0.31
9-Methyluric acid, 9-MU	0.43
Xanthine, X	0.62
7-Methyluric acid, 7-MU	0.71
3-Methyluric acid, 3-MU	0.72
9-Methylxanthine, 9-MX	0.80
1-Methyluric acid, 1-MU	1.10
7,9-Dimethyluric acid, 7,9-MU	1.12
1,9-Dimethyluric acid, 1,9-MU	1.42
3,7-Dimethyluric acid, 3,7-MU	1.68
7-Methylxanthine, 7-MU	1.83
3,9-Dimethylxanthine, 3,9-MX	1.99
3-Methylxanthine, 3-MX	2.46
1,9-Dimethylxanthine, 1,9-MX	2.75
1,3-Dimethyluric acid, 1,3-MU	2.95
1-Methylxanthine, 1-MX	3.00
1,7-Dimethyluric acid, 1,7-MU	3.39
3,7,9-Trimethyluric acid, 3,7,9-MU	4.39
3,7-Dimethylxanthine, 3,7-MX	5 21
1,3,9-Trimethyluric acid, 1,3,9-MU	5.87
1,3,9-Trimethylxanthine, 1,3,9-MX	7 20
1,3,7-Trimethyluric acid, 1,3,7-MU	7.78
1,7-Dimethylxanthine, 1,7-MX	8.00
1,3-Dimethylxanthine, 1,3-MX	9.16
1,3,7,9-Tetramethyluric acid, 1,3,7,9-MU	18.44
1,3,7-Trimethylxanthine, 1,3,7-MX	29.00

Statistical analysis was carried out by means of the Wilcoxon sum of rank test and by the Kruskall–Wallis test [8].

RESULTS

Table I gives the capacity factors of all the xanthines investigated. The effects of the substituents of the xanthine skeleton on k' are as follows.

The 1-methyl group increases the capacity factor by a group contribution value of 4.26 in the xanthines and by a value of 3.98 in the hydroxylated xanthines, the urates ($p=0.5745$); the average value is 4.12 (Table II). Substitution of a methyl group at the 3 position shows an increase in the capacity factor by a factor value of 3.10 in the xanthines and by a value of 2.95 in the urates ($p=0.4712$); the average factor value is 3.03 (Table III). Substitution of a methyl group at the 7 position shows a comparable factor value of 2.73 in the xanthines and a factor value of 2.68 in the urates ($p=0.7491$); the average factor is 2.70 (Table IV).

TABLE II

INFLUENCE OF THE 1-METHYL SUBSTITUENT IN XANTHINES

Compound*	k'	Ratio k' group contribution factor
1-MX	3.00	
X	0.62	4.84
1,3-MX	9.16	
3-MX	2.46	3.72
1,7-MX	8.00	
7-MX	1.83	4.37
1,3,7-MX	29.00	
3,7-MX	5.21	5.57
1,9-MX	2.75	
9-MX	0.80	3.44
1,3,9-MX	7.20	
3,9-MX	1.99	3.62
		4.26 ± 0.83
1-MU	1.10	
U	0.31	3.55
1,3-MU	2.95	
3-MU	0.72	4.10
1,7-MU	3.39	
7-MU	0.71	4.10
1,3,7-MU	7.78	
3,7-MU	1.68	4.63
1,9-MU	1.42	
9-MU	0.43	3.30
1,3,7,9-MU	18.44	
3,7,9-MU	4.39	4.20
		3.98 ± 0.48
All		4.12 ± 0.66
		Prob. $> Z = 0.5745$ NS

*For abbreviations, see Table I.

Substitution of methyl group at the 9 position shows a significantly higher ($p = 0.0142$) factor value for urates (2.24) than for xanthines (1.07) (Table V).

The overall effect of C-8 hydroxylation on the chromatography of xanthines and urates is a reduction in the capacity factor. It is reduced by a factor value of 3.34 in the 3-methyl xanthines, by a value of 2.42 in the 1,7-dimethylxanthines and by a factor value of 1.68 in 9-methylxanthines (Table VI).

The 3-methyl group enhances the reductive effect of the C-8 hydroxyl group with a factor value of 1.39 ($3.34/2.42$; $p = 0.0304$) in comparison with the 1-methyl and/or the 7-methyl group. The 1-methyl and 7-methyl groups enhance the re-

TABLE III

INFLUENCE OF THE 3-METHYL SUBSTITUENT IN XANTHINES

Compound*	k'	Ratio k' group contribution factor
3-MX	2.46	
X	0.62	3.97
1,3-MX	9.16	
1-MX	3.00	3.05
3,7-MX	5.21	
7-MX	1.83	2.85
1,3,7-MX	29.00	
1,7-MX	8.00	3.63
3,9-MX	1.99	
9-MX	0.80	2.49
1,3,9-MX	7.20	
1,9-MX	2.75	2.62
		3.10 ± 0.58
3-MU	0.72	
U	0.31	2.32
1,3-MU	2.95	
1-MU	1.10	2.68
3,7-MU	1.68	
7-MU	0.71	2.37
1,3,7-MU	7.78	
1,7-MU	3.39	2.29
1,3,9-MU	5.87	
1,9-MU	1.42	4.13
3,7,9-MU	4.39	
7,9-MU	1.12	3.92
		2.95 ± 0.85
All		3.03 ± 0.70
		Prob.$>Z=0.4712$ NS

*For abbreviations, see Table I.

ductive effect with a factor value of 1.44 (2.42/1.68; $p=0.0518$) in comparison with the 9-methyl group.

DISCUSSION

N-Methyl substitution in xanthines results in an increase in lipid solubility and an increase of the capacity factor in a reversed-phase system, as shown by Gaspari and Bonati [9]. The factor value of the capacity factors reflects the in-

TABLE IV

INFLUENCE OF THE 7-METHYL SUBSTITUENT IN XANTHINES

Compound*	k'	Ratio k' group contribution factor
7-MX	1.83	
X	0.62	2.95
1,7-MX	8.00	
1-MX	3 00	2.67
3,7-MX	5.21	
3-MX	2.46	2.12
1,3,7-MX	29.00	
1,3-MX	9.16	3.17
		2.73 ± 0.45
7-MU	0.71	
U	0.31	2.29
1,7-MU	3.39	
1-MU	1.10	3.08
3,7-MU	1.68	
3-MU	0.72	2.33
1,3,7-MU	7.78	
1,3-MU	2.95	2.64
7,9-MU	1.12	
9-MU	0.43	2.60
1,3,7,9-MU	18.44	
1,3,9-MU	5.87	3.14
		2.68 ± 0.36
All		2.70 ± 0.38
		Prob. $> Z = 0.7491$ NS

*For abbreviations, see Table I.

crease of lipid solubility. The descending order of the effect of N-methyl substitution is 1-methyl > 3-methyl > 7-methyl > 9-methyl. A similar ranking was observed for the additive group contribution for lipid solubility the "pi (π) values" of the methyl groups [9,10]. N-Demethylation of methyl-substituted xanthines results in an increase in pK_a value. The pK_a value of monomethylxanthines varies between 8 and 9, and that of dimethyl- and trimethylxanthines between 9 and 14. The pK_a value decreases with C-8 hydroxylation; the pK_a of 1,3,7-MX is 14.0, and that of 1,3,7-trimethyluric acid is 6.0 [9,11].

The 1-, 3-, and 7-methyl groups did not show any difference between the capacity factors of xanthines and urates. So, the increase in lipid solubility and retention time due to the three methyl groups is much more important than the increase in water solubility and reduction of the capacity factor due to the C-8 hydroxyl group [9,10]. The effect of the 3-methyl group and the C-8 hydroxyl group is clear from the data in Table VI.

TABLE V

INFLUENCE OF THE 9-METHYL SUBSTITUENT IN XANTHINES

Compound*	k'	Ratio k' group contribution factor
9-MX	0.80	
X	0.56	1.43
1,9-MX	2.75	
1-MX	2.68	1.03
3,9-MX	1.99	
3-MX	2.16	0.92
1,3,9-MX	7.20	
1,3-MX	8.16	0.88
		1.07 ± 0.25
9-MU	0.43	
U	0.21	2.05
1,9-MU	1.42	
1-MU	0.84	1.69
7,9-MU	1.12	
7-MU	0.63	1.78
1,3,9-MU	5.87	
1,3-MU	2.40	2.45
3,7,9-MU	4.39	
3,7-MU	1.54	2.85
1,3,7,9-MU	18.44	
1,3,7-MU	7.10	2.60
		2.24 ± 0.47
		Prob. $> Z = 0.0142$ S

*For abbreviations, see Table I.

In a well defined system of mobile phase and solute, a series of structurally related compounds will always show a fixed ratio in capacity factor when no active binding processes are involved. This has been shown previously for acetylated and hydroxylated sulphonamides [12,13], for a large series of cannabinoids [14], for conjugated bile acids [15,16] and for β-adrenoceptor blocking agents [17]. With this k' ratio it is possible to "identify", or at least become aware of, structurally related compounds or metabolites. In this way, hydroxy- and carboxysulphonamides have been identified in the plasma and urine of humans and animals [13], and cannabinoids with a smaller alkyl side-chain were discovered [18]. In patients without kidney function, or on continous ambulatory peritoneal dialysis, it is possible to identify the series of endogenous and exogenous xanthines (caffeine, theobromine) and their metabolites (to be published). In this way it must also be possible to "identify" conjugates (glucuronides, acetates, sulphates, glycinates, etc.) of drugs and their corresponding metabolites.

TABLE VI

INFLUENCE OF THE C-8 HYDROXYL SUBSTITUENT IN XANTHINES

Compound*	k'	Ratio k' group contribution factor	
3-MX	2.46		
3-MU	0.72	3.42	
1,3-MX	9.16		
1,3-MU	2.95	3.11	
3,7-MX	5.21		
3,7-MU	1.68	3.10	
1,3,7-MX	29.00		
1,3,7-MU	7.78	3.73	
		3.34 ± 0.30	$3.34/2.41 = 1.39$ $p = 0.0304$
X	0.62		
U	0.31	2.00	
1-MX	3.00		
1-MU	1.10	2.73	
7-MX	1.83		
7-MU	0.71	2.58	
1,7-MX	8 00		
1,7-MU	3.39	2.36	
		2.41 ± 0.32	$2.41/1.68 = 1.44$ $p = 0.0518$
9-MX	0.80		
9-MU	0.43	1.68	
1,9-MX	2.75		
1,9-MU	1.42	1.94	
1,3,9-MX	7.20		
1,3,9-MU	5.87	1.23	
		1.68 ± 0.39	

*For abbreviations, see Table I.

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