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Isotopic effects on retention times of caffeine and its metabolites 1,3,7-trimethyluric acid, theophylline, theobromine and paraxanthine

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Abstract

Physicochemical parameters that influence gas chromatographic separation are numerous. Consequently, isotope labelling, because it modifies physicochemical properties, can induce isotopic effects on retention time. Caffeine has been chosen to study this influence because as itself and its metabolites, it allows the preparation of different methylxanthine isotopomers and thus is one of the best models to study isotopic effects induced by stable isotope labelling. Using a caffeine molecule labelled with deuterium at different positions and rat hepatocytes to obtain metabolites, it was possible to study the influence of labelling on retention time [(14% cyanopropylphenyl)methylpolysiloxane] and to point out the role of each labelled site. It appears that isotopic effects induced by the labelling depend not only on the number of labelling atoms but also on whether this labelling is at position 1, 3 or 7 and, consequently, on the role of the labelled site on the function of the molecule.

Keywords: Caffeine; 1,3,7-Trimethyluric acid; Dimethylxanthines; Theophylline; Theobromine; Paraxanthine

1. Introduction

The use of stable isotope labelling in the study of drug metabolism provides useful information on the in vivo behaviour of parent compounds and their metabolites, as well as on their mechanisms of formation. Caffeine is metabolised by the liver through C-oxidation and N-demethylation reactions catalysed by xanthine oxidase and cytochrome P450. Thanks to the different metabolic pathways occurring in caffeine and other methylxanthine metabolism and the number of different isomers which can be obtained, this family can be used as a pertinent model to study the influence of labelling and particularly, the influence of labelling on physicochemical properties.

Bechalany et al. [1] using HPLC, demonstrated that when CH_3 groups are substituted with deuterium, the lipophilicity of caffeine isotopomers decreases and that specific isotopic effects are induced according to the labelling position. Cherrah et al. [2] performed a study on the binding of caffeine and its deuterated isotopomers to human serum albumin. Important and significant isotope effects appeared on the various binding parameters. In the same way, they demonstrated that protein binding parameters are also greatly affected when using competitive coupling of unlabelled and labelled molecules: theophylline/1-C(^{2}H)$_3$-theophylline, phenobarbital/5-(^{2}H)$_5$-phenyl-phenobarbitone, phen-

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obarbital/1,3-$^{15}N_2$,2-^{13}C-phenobarbital [3]. Nevertheless, deuterium substitution on the five hydrogen atoms of the ethyl group did not induce any isotope effect on protein binding. Metabolic and kinetic isotope effects were also demonstrated with caffeine [4–7]. So, it seemed interesting to study the influence of deuteration on the physicochemical properties and particularly on GC retention times using the various isotopomers of caffeine and of its metabolites. Consequently, all the deutero-isotopomers of caffeine (TMX) corresponding to the labelling of the various methyl groups at N1, N3 and N7 were used, as well as the corresponding dimethylxanthine (DMX) and trimethyluric acid (TMU) metabolites prepared, in vitro, from rat hepatocytes. Thus, it was possible to measure isotopic effects on GC retention times and to determine the relative influence of the number of labelling atoms and of their respective location.

2. Experimental

2.1. Chemicals

Labelled caffeine molecules were synthesised as previously described [8]. Unlabelled caffeine, 3-isobutyl-1-methylxanthine were obtained from Sigma (St Louis, MO, USA). 1,3-$^{15}N_2$,2-^{13}C-caffeine from CEA (Saclay, France). Collagenase was obtained from Boehringer (Mannheim, Germany) and sodium pentobarbital from Clin Midy (Paris, France). All other chemicals were of analytical grade and obtained from local commercial sources.

2.2. Preparation of caffeine metabolites

Preparation of hepatocytes, isolated from adult male Wistar rats, and caffeine isotopomer incubation were carried out according to the technique previously described [4]. The various demethylated metabolites of caffeine, as well as trimethyluric acid isotopomers, were obtained from these incubation media and analysed by capillary gas chromatography–mass spectrometry. The chemical structure of the studied compounds and the m/z value of the ions used for their GC–MS monitoring are given in Table 1.

2.3. Extraction and derivatization

Perchloric acid (100 μl, 12%), 500 mg of ammonium sulfate, 100 μl of internal standard solutions [(1,3-$^{15}N_2$,2-^{13}C-caffeine (20 mg/l) and 3-isobutyl-1-methylxanthine (10 mg/l)], 300 μl of distillated water and 10 ml of chloroform–isopropanol (85:15, v/v) were added to 500 μl of biological samples in a 20-ml glass tube and stirred for 10 min. Aqueous and organic layers were separated by centrifugation (3 min, 2000 *g*). The organic phase was evaporated to dryness in a 15-ml glass tube at room temperature under a stream of nitrogen.

N,N-Dimethylacetamide (200 μl) was added to the dry residue and stirred for 15 s. Then, 100 μl of tetraethylammonium hydroxide was added and stirred for 15 s followed by 120 μl of iodobutane which was stirred again for 15 s. The solution was then left for one night at room temperature. After centrifugation (5 min, 2000 *g*), the supernatant was evaporated to dryness at room temperature under nitrogen and the residue reconstituted with 100 μl of a toluene–ethyl acetate mixture (85:15, v/v) for GC–MS analysis.

2.4. Gas chromatography–mass spectrometry

GC–MS analysis [9] was carried out using a HP 5890 gas chromatograph and a HP 5970 mass spectrometer. The mass spectra were obtained under electron impact (70 eV). The interface was maintained at 260°C.

Chromatographic separations were performed using a OV 1701 column [25 m×0.25 mm I.D., film thickness 0.20 μm, (14% cyanopropylphenyl)-methylpolysiloxane]. The oven temperature was held at 110°C for 0.8 min and then programmed from 110 to 280°C at 12.5°C/min. The injector was held at 260°C. The extracts were injected in the splitless mode. The injected volume was 1 μl.

2.5. Isotopic effect measurement and expression

The following procedure was used in order to measure the isotopic effect induced by deuterium on the retention times:

1. Injection of a mixture containing the unlabelled

Table 1
Isotopomers of caffeine and their potential N-demethylated and C-oxidised metabolites

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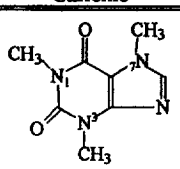	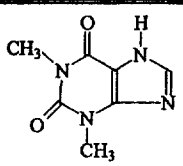	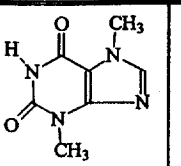	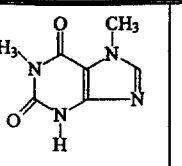	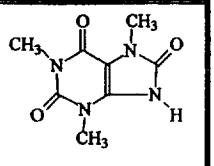
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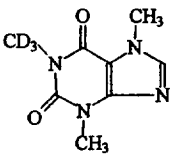	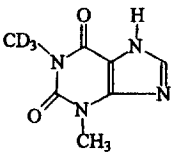	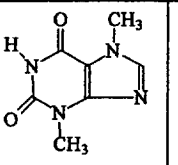	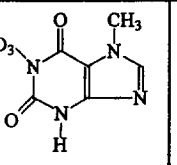	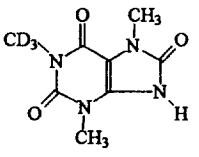
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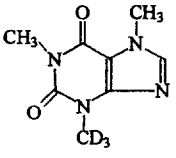	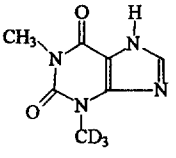	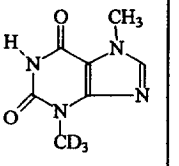	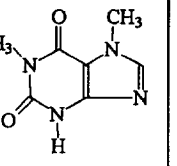	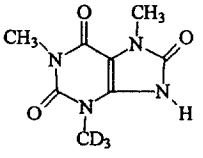
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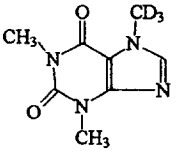	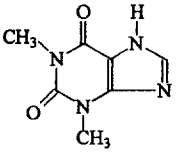	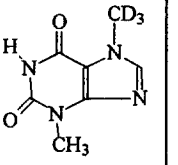	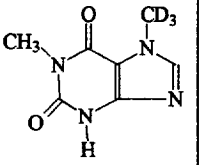	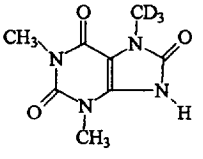
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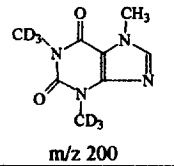	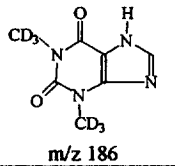	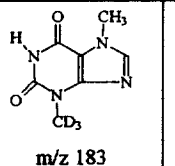	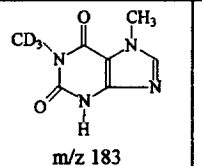	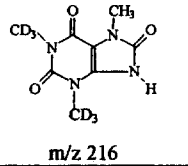
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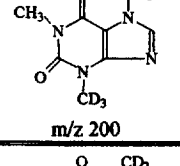	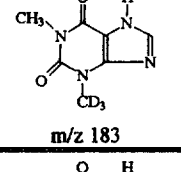	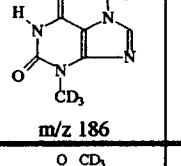	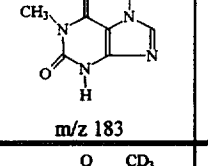	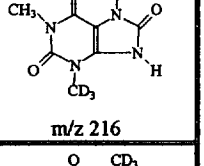
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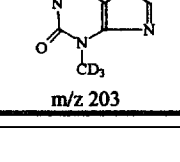	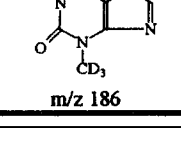	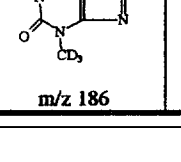	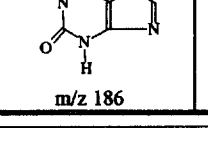	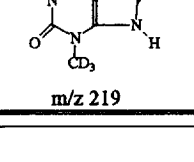
m/z 203	m/z 186	m/z 186	m/z 186	m/z 219

Table 2
Isotopic effects on relative retention times of caffeine

Caffeine isotopomers	n	Mean	S.D.	Maximum	Minimum
1-C^2H_3	30	1.00266	$2.21 \cdot 10^{-4}$	1.00316	1.00230
3-C^2H_3	30	1.00260	$3.08 \cdot 10^{-4}$	1.00321	1.00210
7-C^2H_3	30	1.00147	$2.90 \cdot 10^{-4}$	1.00213	1.00106
1,3-C^2H_3	30	1.00490	$2.20 \cdot 10^{-4}$	1.00536	1.00447
3,7-C^2H_3	30	1.00398	$2.91 \cdot 10^{-4}$	1.00435	1.00304
1,7-C^2H_3	30	1.00411	$2.61 \cdot 10^{-4}$	1.00457	1.00354
1,3,7-C^2H_3	30	1.00668	$2.54 \cdot 10^{-4}$	1.00732	1.00628

molecule and its labelled counterpart with the internal standards: 1,3-$^{15}N_2$,2-^{13}C-caffeine for labelled and unlabelled caffeine isotopomers and 3-isobutyl-1-methylxanthine for metabolites.

2. Measurement of the retention time relative to the appropriate internal standard: Tr_{d0}=unlabelled compound relative retention time and Tr_{iso}= isotopomer relative retention time.
3. The expression of the isotopic effect was: $IE = Tr_{d0}/Tr_{iso}$.

This procedure was repeated 30 times for each TMX and DMX isotopomers and 24 times for TMU isotopomers. The increment of isotope effect afforded by each (–C^2H_3) substitution was calculated as the difference of the stable isotope effect values of each isotopomer and expressed as *IED* (isotope effect difference). These increments were compared using the Student t-test (unpaired) with a significant level at $p<0.05$.

3. Results and discussion

3.1. Caffeine isotopomers

The mean values (±S.D.) of isotopic effects for each deuterated caffeine isotopomer are given in Table 2. It can be seen from these results, that the magnitude of the isotope effect induced by deuterium substitution increases with the number of deuterium atoms introduced into the caffeine molecule. Fig. 1 shows a chromatogram of deuterated caffeines.

A more precise analysis can be performed by the calculation of the increments of isotope effect afforded by the substitution at N1, N3 or N7 positions. Table 3 shows the influence of the first substitution for the three mono-trideuteromethyl isotopomers of caffeine. There is no significant difference in the increments of isotope effect between the N1 and N3 substitutions. The isotope effect corresponding to the N7 substitution is significantly lower than those observed both at the N3 and N1 positions.

Tables 4–6 display the values of the increments of isotope effects induced by N1, N3 and N7 substitutions, respectively, for all the couples of mono, di and trideuteromethyl caffeine isotopomers.

The observation of the data shows that when comparing the three mono-deuteromethyl isotopomers of caffeine, the isotope effect on retention times induced by N1 and N3 substitutions are not significantly different. N7 substitution has a significantly lower effect. For the di-deuteromethyl isotopomers, the effect of substitution either at N1 or at N3 differs according to the position of the first labelling site (N3 or N7 for N1 substitution, N1 or

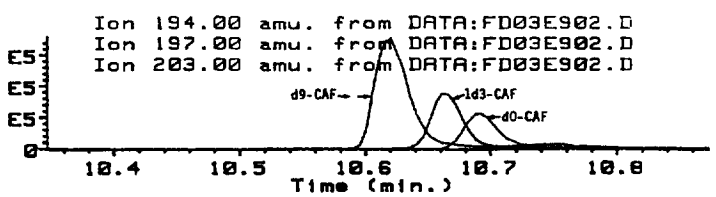

Fig. 1. Chromatogram of caffeine and two of its isotopomers.

Table 3
First substitution influence

	Confidence interval (95%)		Difference mean	Student test		
	(×1000)		(×1000)	(×1000)		
0 → 1	2.5764	2.7414	2.6589	(0 → 1) vs. (0 → 3)	−0.060	$p>0.05$
0 → 3	2.4837	2.7136	2.5986	(0 → 1) vs. (0 → 7)	−1.187	$p<0.001$
0 → 7	1.3662	1.5817	1.4719	(0 → 3) vs. (0 → 7)	−1.127	$p<0.001$

Table 4
Influence of substitution on 1

	Confidence interval (95%)		Difference mean	Student test		
	(×1000)		(×1000)	(×1000)		
0 → 1	2.5764	2.7414	2.6589	(0 → 1) vs. (3 → 1,3)	−0.3602	$p<0.001$
				(0 → 1) vs. (7 → 1,7)	−0.0197	$p>0.05$
3 → 1,3	2.1603	2.4370	2.2987	(0 → 1) vs. (3,7 → 1,3,7)	0.0379	$p>0.05$
7 → 1,7	2.4955	2.7829	2.6392	(3 → 1,3) vs. (7 → 1,7)	0.3405	$p<0.001$
				(3 → 1,3) vs. (3,7 → 1,3,7)	0.3981	$p<0.001$
3,7 → 1,3,7	2.5557	2.8380	2.6968	(7 → 1,7) vs. (3,7 → 1,3,7)	0.0576	$p>0.05$

Table 5
Influence of substitution on 3

	Confidence interval (95%)		Difference mean	Student test		
	(×1000)		(×1000)	(×1000)		
0 → 3	2.4837	2.7136	2.5986	(0 → 3) vs. (1 → 1,3)	−0.3602	$p<0.001$
				(0 → 3) vs. (7 → 3,7)	−0.0891	$p>0.05$
1 → 1,3	2.1243	2.3524	2.2384	(0 → 3) vs. (1,7 → 1,3,7)	−0.0295	$p>0.05$
7 → 3,7	2.3583	2.6609	2.5095	(1 → 1,3) vs. (7 → 3,7)	0.2711	$p<0.001$
				(1 → 1,3) vs. (1,7 → 1,3,7)	0.3307	$p<0.001$
1,7 → 1,3,7	2.4340	2.7002	2.5691	(7 → 3,7) vs. (1,7 → 1,3,7)	0.0596	$p>0.05$

Table 6
Influence of substitution on 7

	Confidence interval (95%)		Difference mean	Student test		
	(×1000)		(×1000)	(×1000)		
0 → 7	1.3621	1.5817	1.4719	(0 → 7) vs. (1 → 1,7)	−0.0197	$p>0.05$
				(0 → 7) vs. (3 → 3,7)	−0.0891	$p>0.05$
1 → 1,7	1.3272	1.5772	1.4522	(0 → 7) vs. (1,3 → 1,3,7)	0.3090	$p<0.001$
3 → 3,7	1.2280	1.5376	1.3828	(1 → 1,7) vs. (3 → 3,7)	−0.0694	$p>0.05$
				(1 → 1,7) vs. (1,3 → 1,3,7)	0.3287	$p<0.001$
1,3 → 1,3,7	1.6581	1.9038	1.7809	(3 → 3,7) vs. (1,3 → 1,3,7)	0.3981	$p<0.001$

Table 7
Isotopic effects on relative retention times of trimethyluric acid

Caffeine isotopomers	n	Mean	S.D.	Maximum	Minimum
1-CD_3	24	1.00218	8.18×10^{-4}	1.00379	1.00077
3-CD_3	24	1.00419	2.77×10^{-4}	1.00196	1.00069
7-CD_3	24	1.00123	3.04×10^{-4}	1.00150	1.00090
1,3-CD_3	24	1.00341	1.56×10^{-4}	1.00371	1.00320
3,7-CD_3	24	1.00264	2.96×10^{-4}	1.00294	1.00153
1,7-CD_3	24	1.00338	2.54×10^{-4}	1.00359	1.00300
1,3,7-CD_3	24	1.00454	4.98×10^{-4}	1.00560	1.00342

N7 for N3 substitution), whereas the effect of the N7 substitution does not depend on the location at N1 or N3 of the first deuteromethyl groups. Here too, the overall effect of N1 or N3 substitution is significantly higher than that observed with the N7 substitution. At last, when two sites are labelled, the substitutions at N1 and N3 do not significantly differ but still have a significantly higher effect than the N7 one.

These results show the particularity of the N7 position and allow us to classify the order of magnitude of isotope effects in caffeine deuteroisotopomers as N1 ≠ N3>N7.

3.2. Trimethyl uric acid isotopomers

The mean values (±S.D.) of isotopic effects for each deuterated trimethyluric acid isotopomer are gathered in Table 7. Like for caffeine isotopomers, the magnitude of the isotope effect induced by deuterium substitution increases with the number of deuterium atoms introduced into the molecule. Table 8 shows the influence of the first substitution and Tables 9–11 the values of the increments of isotope effect induced by N1, N3 and N7 substitutions, respectively, for all the couples of mono, di and tri deuteromethyl uric acids. This data shows that the isotope effect is significantly different according to the site of substitution in mono deuteromethyl uric acids. The order of magnitude is N1>N3>N7. In comparison to the substitution at N3 in caffeine isotopomers, the increment of isotope effect is lower in TMU and there is a difference between the N1 and N3 sites. For the dideuteromethyl isotopomers, the effect of substitution both at N1 and N7 differs

Table 8
First substitution influence

	Confidence interval (95%)		Difference mean	Student test		
	(×1000)		(×1000)	(×1000)		
0 → 1	1.8320	2.5234	2.1777	(0 → 1) vs. (0 → 3)	−0.6860	$p<0.001$
0 → 3	1.3745	1.6089	1.4917	(0 → 1) vs. (0 → 7)	−0.9429	$p<0.001$
0 → 7	1.1611	1.3085	1.2348	(0 → 3) vs. (0 → 7)	−0.2569	$p<0.01$

Table 9
Influence of substitution on 1

	Confidence interval (95%)		Difference mean	Student test		
	(×1000)		(×1000)	(×1000)		
0 → 1	1.8320	2.5234	2.1777	(0 → 1) vs. (3 → 1,3)	−0.2577	$p>0.05$
				(0 → 1) vs. (7 → 1,7)	−0.0277	$p>0.05$
3 → 1,3	1.7917	2.0484	1.9200	(0 → 1) vs. (3,7 → 1,3,7)	−0.2768	$p>0.05$
7 → 1,7	2.0181	2.2622	2.1500	(3 → 1,3) vs. (7 → 1,7)	0.2300	$p<0.001$
				(3 → 1,3) vs. (3,7 → 1,3,7)	−0.0191	$p>0.05$
3,7 → 1,3,7	1.6625	2.1393	1.9009	(7 → 1,7) vs. (3,7 → 1,3,7)	−0.2491	$p>0.05$

Table 10
Influence of substitution on 3

	Confidence interval (95%)		Difference mean	Student test		
	(×1000)		(×1000)	(×1000)		
0 → 3	1.3745	1.6089	1.4917	(0 → 3) vs. (1 → 1,3)	−0.2577	$p<0.001$
				(0 → 3) vs. (7 → 3,7)	−0.0888	$p>0.05$
1 → 1,3	1.7892	2.0508	1.2340	(0 → 3) vs. (1,7 → 1,3,7)	−0.3317	$p<0.01$
7 → 3,7	1.2615	1.5444	1.4029	(1 → 1,3) vs. (7 → 3,7)	0.1689	$p<0.02$
				(1 → 1,3) vs. (1,7 → 1,3,7)	−0.0740	$p>0.05$
1,7 → 1,3,7	0.8619	1.4654	1.1600	(7 → 3,7) vs. (1,7 → 1,3,7)	−0.2429	$p<0.05$

according to the location of the first labelling group, whereas, N3 substitution does not display any significant difference whether the N1 site or the N7 site are previously labelled. Here too, the overall effect is N1>N3>N7. The same order is observed for the substitution of the last site when two sites are already labelled.

3.3. Dimethylxanthines isotopomers: deuteromethylxanthine, dideuteromethylxanthine (theophylline, theobromine, paraxanthine)

Table 12 gathers the values of the isotope effect observed on the retention times of the various deuteromethylmethylxanthine as well as their comparisons ($p<0.001$). As for TMX and TMU, the isotope effect induced by deuterium substitution at N1 or N3 is significantly higher than that induced by N7 substitution. Moreover, there is a small but significant difference in the isotope effect observed in the N(3)–C^2H_3 isotopomer according to the location at N1 or N7 of the second methyl group. So the order of magnitude of this effect is N1 # N3>N7.

The statistical comparison of the retention times of the three couples of deuteromethylxantines (1–3, 1–7, 3–7) according to the relative positions of the CH_3 group and of the C^2H_3 group, shows that for each couple, the retention times are significantly

Table 12
Influence of first substitution on dimethylxanthines

1	3	7	ΔIE	Student test
C^2H_3	CH_3		2.229×10^{-3}	$p<0.01$
C^2H_3		CH_3	1.977×10^{-3}	
CH_3	C^2H_3		2.143×10^{-3}	$p<0.001$
	C^2H_3	CH_3	2.358×10^{-3}	
CH_3		C^2H_3	1.259×10^{-3}	$p<0.05$
	CH_3	C^2H_3	1.215×10^{-3}	

$\Delta IE = IE(d0) - IE(CD_3)$.

Table 11
Influence of substitution on 7

	Confidence interval (95%)		Difference mean	Student test		
	(×1000)		(×1000)	(×1000)		
0 → 7	1.1611	1.3085	1.2348	(0 → 7) vs. (1 → 1,7)	−0.0348	$p>0.05$
				(0 → 7) vs. (3 → 3,7)	−0.0888	$p>0.05$
1 → 1,7	0.7091	1.6855	1.2000	(0 → 7) vs. (1,3 → 1,3,7)	−0.1079	$p>0.05$
3 → 3,7	0.9791	1.3129	1.1460	(1 → 1,7) vs. (3 → 3,7)	−0.0540	$p>0.05$
				(1 → 1,7) vs. (1,3 → 1,3,7)	−0.0731	$p>0.05$
1,3 → 1,3,7	0.9122	1.3415	1.1269	(3 → 3,7) vs. (1,3 → 1,3,7)	−0.0191	$p>0.05$

Table 13
Influence of CH_3/C^2H_3 positions in mono-trideuteromethylated dimethylxanthines

1	3	7	IE	Confidence interval (95%)		Student test
CH_3		C^2H_3	1.00198	5.050×10^{-4}	9.280×10^{-4}	$p<0.001$
C^2H_3		CH_3	1.00126			
	CH_3	C^2H_3	1.00236	0.978×10^{-4}	1.306×10^{-4}	$p<0.001$
	C^2H_3	CH_3	1.00122			
CH_3	C^2H_3		1.00223	1.440×10^{-4}	3.150×10^{-4}	$p<0.001$
C^2H_3	CH_3		1.00214			

different from one isotopomer to the other (Table 13). These differences are due to inequivalent isotope effects occurring by substitution of the various methyl groups and to their respective combinations.

Table 14 displays the values of the isotope effect measured from the gas chromatographic analysis of the various isotopomers of all the deuteromethylmethylxanthines and dideuteromethylxanthines. It can be observed that when a second deuteromethyl group is substituted to a methyl group in the various deuteromethylmethyl xanthine molecules, there is a significantly increased isotope effect. The inequivalence of the various sites is still observed with a larger effect on sites N1 and N3 than on site N7. It can be also concluded that for the second substitution of dimethylxanthines, N1 # N3>N7. Here too, the effect at N1 is larger than at N3, but not significantly. Whatever the first or the second C^2H_3 substitution, the increment of isotope effect afforded by N7 substitution is always one half the value given either by N1 or N3 substitutions. The values of the increments in isotope effect are slightly lower (but not significantly) at all positions for the second deuteromethyl substitution.

4. Conclusions

All the possible combinations of the deuteroisotopomers of trimethyl uric acids and dimethylxanthines were obtained by the synthesis of the various deuteromethyl isotopomers of caffeine (TMX) and their incubation with rat hepatocytes. The GC–MS analysis in the selected ion monitoring

Table 14
Isotopic effect and confidence interval (95%) of isotopic effect differences from various dimethylxanthines

Student test	Confidence interval (95%) ($\times10^4$)		Isotopic effect	1	2	3	Isotopic effect	Confidence interval (95%) ($\times10^3$)		Student test
$p<0.01$	0.1198	5.1692	1.00223	C^2H_3	CH_3		1.00223	1.585	2.0234	$p<0.001$
			1.00198	C^2H_3		CH_3	1.00198	1.1895	1.5721	$p<0.001$
$p<0.01$	5.47	7.98	1.00403	C^2H_3	C^2H_3		1.00403			
			1.00336	C^2H_3		C^2H_3	1.00336			
$p<0.001$	0.514	3.780	1.00236		C^2H_3	CH_3	1.00236	0.598	0.992	$p<0.001$
			1.00214	CH_3	C^2H_3		1.00214	1.7496	2.0304	$p<0.001$
$p<0.001$	0.701	1.058	1.00315		C^2H_3	C^2H_3	1.00315			
			1.00403	C^2H_3	C^2H_3		1.00403			
$p>0.05$	−1.24	2.11	1.00126	CH_3		C^2H_3	1.00126	1.94	2.25	$p<0.001$
			1.00122		CH_3	C^2H_3	1.00122	1.74	2.13	$p<0.001$
$p<0.001$	0.253	3.87	1.00336	C^2H_3		C^2H_3	1.00336			
			1.00315		C^2H_3	C^2H_3	1.00315			

mode (SIM) permitted the accurate measurement of the retention times of all these labelled and unlabelled compounds. The comparison between the retention time of the unlabelled compound and those of its various isotopomers gave the values of the isotope effect induced by deuterium labelling. It can be observed that for TMX, TMU and DMX isotopomers, the isotope effect increases proportionally with the number of deuterium atoms introduced into the molecule: 1.0022 (d3-TMX), 1.0044 (d6-TMX), 1.0067 (d9-TMX), 1.0016 (d3-TMU), 1.0031 (d6-TMU), 1.0045 (d9-TMU), 1.0018 (d3-DMX) and 1.0035 (d6-DMX).

A more precise analysis shows that the isotope effect not only depends on the number of deuterium atoms in the molecule but also on the location of the labelling. As the three N–CH_3 groups on the xanthine ring are not chemically equivalent, there is a difference in the increment of isotope effect, whether the substitution takes place at the N1, N3 or N7 position.

The isotope effect induced by the N7 substitution is always significantly lower than those obtained after N1 or N3 labelling which give nearly the same isotope effect. The progressive deuterosubstitution of the three methyl groups of the xanthine ring points out the inequivalence of these N–CH_3 groups. It also confirms the peculiar behaviour of the N(7)–CH_3 site. This observation is in agreement with previously reported data.

The peculiar behaviour of the N(7)–CH_3 site was also previously observed [10–12]. Indeed, N(7) methylation has no influence on lipophilicity, contrary to N(1) methylation [10]. Moreover, in human metabolism, N(7) demethylation (4%) is lower than N(1) demethylation (12%) and N(3) demethylation (84%) [11]. Concerning acidity, it appears that theophylline and paraxanthine pK_a are quite identical, even though theobromine is superior [12]. Once more the particularity of the N(7) site is demonstrated.

A deuterosubstitution can have an influence on: the vaporisation and the phenomenons linked with the vapour pressure, the repartition and the diffusion of the analyte between both phases. In gas chromatography, it is possible to consider that the analyte is always diluted in solvent molecules. Thus, from an energetic aspect, the analyte molecule has to overcome the energy resulting from the actions of the solvent molecules. Then, the relative volatility of the molecules of two different analytes (A and B) diluted in a solvent (S) is measured by the difference between the energies: $\Sigma(E_A-E_S)-\Sigma(E_B-E_S)$. Consequently, all the factors that can modify $\Sigma(E_A-E_S)$ or $\Sigma(E_B-E_S)$ will modify the relative chromatographic parameters of both analytes. Thus, concerning the deuterosubstitution, the differences of molecular volume, of polarisability and of mass between hydrogen and deuterium will modify the retention times, as these parameters have an influence on the energies.

This study on the overall and local effect of deuterosubstitution on inequivalent N–CH_3 groups confirms that by decreasing the molar volume, deuterium substitution decreases the lipophilicity of the TMX, DMX and TMU molecules, which is one of the factors influencing molecular interactions with the GC stationary phase and thus retention times. It also shows that this variation is modulated according to the location of the substitution. As a consequence, when a molecule has to be labelled with deuterium for tracing purposes, a careful choice has to be made, not only on the number but also on the precise location of the labelling atoms in order to avoid or lessen isotope effects. These results also illustrate the use of successive isotopic molecular perturbations as a methodology, allowing us to study the functionality or the implication of the various molecular sites in a given physicochemical or biological property of the molecule.

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