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Gas chromatographic–mass spectrometric quantitation of tri-, di- and monomethylxanthines and uric acids from hepatocyte incubation media

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ABSTRACT

A gas chromatographic–mass spectrometric method is described for the measurement of the concentration of fourteen methylxanthines and methyluric acid metabolites of methylxanthines, especially caffeine, from cell incubation media. The method shows linearity, accuracy and recovery suitable for metabolic studies The reproducibility of relative retention times is satisfactory (<0.07%) and allows rapid and conclusive identification of chromatographic peaks corresponding to metabolites. Moreover, this method enables the simultaneous determination of 3,7-methylxanthine and its 1,7-isomer, which are not chromatographically resolved This method can be successfully applied when molecules labelled with stable isotopes are used as tracers for metabolic studies.

INTRODUCTION

Methylxanthines are widely studied molecules. These compounds, especially caffeine (1,3,7-trimethylxanthine), are heavily consumed in food and beverages [1], and in addition they are used in clinical medicine as central nervous system stimulants, bronchodilatators or diuretics [2–4]. Caffeine is also used as a metabolic probe for the exploration of oxidative metabolic pathways and the numerous factors that can potentially alter these enzymic activities [5–7]. Demethylation reactions and/or oxidation of C-8 lead to the production of di- and monomethylxanthines (MX) and tri-, di-, and monomethyluric acids (MU), as well as ring-opened uracil-related metabolites [8–10]. The oxidative biotransformation of caffeine occurs primarily in the liver via the microsomal cytochrome P450 monoxygenases [8–10]. Many metabolic studies are now performed using isolated human or animal hepatocytes or cell cultures [11–15]. In order to analyse methylxanthine metabolites from such incubation or culture media, we have developed a

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specific gas chromatographic–mass spectrometric (GC–MS) method to monitor fourteen potential MX and MU metabolites of methylxanthines. It has proved especially suitable when molecules labelled with stable isotopes are used for biological studies, such as the effect of hormones and xenobiotics, drug interactions and enzymic inhibition or induction.

EXPERIMENTAL

Reagents and chemicals

Caffeine (1,3,7-trimethylxanthine, CAF), theophylline (1,3-dimethylxanthine, 1,3MX), paraxanthine (1,7-dimethylxanthine, 1,7MX), theobromine (3,7-dimethylxanthine, 3,7MX), 1-methylxanthine (1MX), 3-methylxanthine (3MX) and 7-methylxanthine (7MX) and their respective methyluric acid analogues, 1,3,7MU (TMU), 1,3MU, 3,7MU, 1,7MU, 1MU, 3MU and 7MU were purchased from Fluka (Buchs, Switzerland).

3-Isobutyl-1-methylxanthine (IBMX) and 1,3-[^{15}N]$_2$,2-[^{13}C]caffeine (CAF*), with an isotopic enrichment of ^{13}C = 99% and ^{15}N = 90%, were obtained from Janssen Chimica (Beerse, Belgium) and Commissariat à l'Energie Atomique (Saclay, France), respectively, and used as internal standards at concentrations of 3 and 5 mg/l, respectively, in methanol.

Tetramethylammonium hydroxide (0.1 *M* in 2-propanol–methanol), N,N-dimethylacetamide (spectro grade), 1-iodobutane and ammonium sulphate were purchased from Merck (Darmstadt, F.R.G.). Chloroform (RPE grade), 2 propanol (RPE-ACS grade), perchloric acid, toluene and ethyl acetate were from Carlo Erba (Milan, Italy).

The hepatocyte incubation medium contained NaCl (118 m*M*), KCl (4.74 m*M*), KH_2PO_4 (1.18 m*M*), $MgSO_4$ (1.18 m*M*), $CaCl_2$ (1.27 m*M*), $NaHCO_3$ (24.88 n*M*), pyruvate (4.4 m*M*), glutamate (4.2 m*M*) and bovine serum albumin (fraction V), 0.25%.

The abbreviations DMX, MMX, DMU and MMU are used for dimethylxanthines, monomethylxanthines, dimethyluric acids and monomethyluric acids respectively.

Standardization

To assess the quality of the analytical method, five spiked standard samples containing caffeine and its metabolites were prepared in the buffer incubation medium The final concentrations were:

Standard 1: caffeine, 0.5 mg/l; 3,7MX and R, 0.25 mg/l; 1,7MX, 0.25 mg/l.
Standard 2: caffeine, 0.5 mg/l; 3,7MX and R, 0.25 mg/l; 1,7MX, 3.00 mg/l.
Standard 3: caffeine, 3.0 mg/l; 3,7MX and R, 1.50 mg/l; 1,7MX, 1.50 mg/l.
Standard 4: caffeine, 6.0 mg/l; 3,7MX and R, 3.00 mg/l; 1,7MX, 0.25 mg/l.
Standard 5: caffeine, 6.0 mg/l; 3,7MX and R, 3.00 mg/l; 1,7MX, 3.00 mg/l.

R represents a solution of all caffeine metabolites except 3,7MX and 1,7MX. These standard solutions can be kept at −20°C until analysis.

Extraction procedure

The internal standard mixture (100 μl), 12% perchloric acid (100 μl), distilled water (500 μl) and ammonium sulphate (500 mg) were added to 500 μl of isolated hepatocytes suspension ($2.5 \cdot 10^6$ cells per ml) in the incubation buffer into a 20-ml screw-cap centrifuge tube. After mixing and addition of 10 ml of the extraction solvent (chloroform–2-propanol, 85:15, v/v), the sample was extracted using a horizontal automatic shaker for 10 min. After centrifugation, the organic phase was transferred to a 15-ml centrifuge tube and evaporated under a stream of nitrogen at 30°C.

Derivatization of N–H groups

The derivatization of N–H groups was performed according to a modification of Greeley's procedure [16,17]. After addition of 200 μl of N,N-dimethylacetamide and 120 μl of tetramethylammonium hydroxide to the dry residue and vigorous shaking for 10 s, 120 μl of 1-iodobutane were added. The solution was shaken again and allowed to stand at room temperature for 12 h. After centrifugation, the organic phase was recovered and evaporated to dryness at 30°C. The dry residue was dissolved in 100 μl of toluene–ethyl acetate (5:2, v/v) for GC–MS analysis.

Temperature was found to be a critical parameter for MU derivatization. The maximum yield was obtained after only 2 min at 70°C but it was followed by a rapid degradation process leading to total disappearance of the product within 30 min. At 50 and 37°C the maximum was obtained after 30–120 min, according to the product to be alkylated. At these temperatures the decomposition rate was proportional to the temperature. When the derivatization was carried out at room temperature (20–25°C) a stable plateau was achieved within 2 h for 1,3MU, 1MU and 3MU, 5 h for TMU and 8 h for 1,7MU, 3,7MU and 7MU at a higher level than with any other of the tested temperatures. Therefore, in order to measure all caffeine metabolites, a reaction at 25°C for at least 8 h is required, *i.e.* overnight at room temperature in practice.

Chromatographic separation

The gas chromatograph was a Hewlett-Packard Model 5790 equipped with an OV 1701 fused-silica capillary column (25 m × 0.25 mm I.D.). Samples (1 μl) were injected according to the splitless mode (valve time, 0.75 min). The injection port temperature was set at 215°C, and the oven temperature was raised from 110°C (0.8 min) to 280°C (2 min) at 12.5°C/min. Helium was used as carrier gas at a linear velocity of 52 cm/s. Head pressure was set at 1 bar.

TABLE I

MONITORING CONDITIONS

Group	Compound	Start time (min)	*m/z*	Dwell time (ms)
1	CAF	10.00	194	50
	CAF*	10.00	197	50
2	DMX	11.45	180	50
	DMX	11 45	136	50
3	IBMX	12 25	178	100
4	TMU	12 75	210	50
	MMX	12.75	278	50
5	DMU	13 25	196	100
6	MMU	14 20	294	100

Detection and measurements

A Hewlett-Packard mass-selective detector (HP 5970B) operating between 10 and 15 min after the run start was used for detection. The transfer line temperature was set at 260°C. In order to measure all the caffeine metabolites, six groups of ions were monitored (Table I).

Mass spectrometer operations and data treatment were under control of an HP 59970 workstation.

RESULTS

The *m/z* values of the major fragments of the perbutylated MX and MU with relative abundances are presented in Tables II and III, respectively. Marked similarities are apparent in the fragmentation pathways of MX and their C-8 oxidized analogues: for each individual fragment ion, a difference of 16 Da relative to the C-8 oxygen atom can be observed, ranging from the dioxo to the homologous trioxo purine molecule. Moreover, the mass spectra of identically methyl-substituted compounds corresponding to the dioxo or the trioxo groups show fragment ions with the same *m/z* value. Compounds can be differentiated by using the distinct relative abundances of their typical ions, which allows a rapid identification of source molecules: *m/z* 136 for 7MX and 1,7MX; *m/z* 178 for 1MX and IBMX; *m/z* 152 and 196 for 7MU; *m/z* 238 for 3MU; and *m/z* 235 for 1,7MU. In order to increase the accuracy of measurements, the choice of the monitored ions was determined by the requirement to follow a minimal number of ions, despite the large number of molecules to be analysed.

Fig. 1 shows a fragmentogram obtained from a hepatocyte suspension incubating buffer spiked with MX and MU (1 mg/l each) under the described chromatographic conditions. Table IV shows the mean values of the absolute and

TABLE II

MASS SPECTRA OF PERBUTYLATED METHYLXANTHINES RELATIVE ABUNDANCES OF MAJOR PEAKS

Compound	*m/z*															
	81	109	122	123	136	166	178	180	193	194	205	207	222	235	236	278
Monomethylxanthines (M_r 166)																
IBMX	3	10	33	–	9	51[a]	100	20	15	–	–	5	76[b]	10	–	21[c]
1MX	8	26	54	–	27	76[a]	88	45	–	12	–	23	100[b]	–	40	84[c]
3MX	15	14	12	28	12	95[a]	6	86	30	14	–	22	89[b]	32	–	100[c]
7MX	17	11	–	28	100	69[a]	4	92	–	10	32	–	51[b]	–	15	98[c]
Dimethylxanthines (M_r 180)																
1,3MX	16	21		32	7			100[a]	–	36		35			99[b]	
1,7MX	14	12		50	100			90[a]	26	42		3			77[b]	
3,7MX	12	67		1	14			100[a]	44	23		12			76[b]	
Trimethylxanthine (M_r 194)																
CAF	29	61			4					100[a]						

[a] Methylxanthine ions

[b] Monobutylmethylxanthine ions

[c] Dibutylmethylxanthine ions

TABLE III

MASS SPECTRA OF PERBUTYLATED METHYLURIC ACIDS: RELATIVE ABUNDANCES OF MAJOR PEAKS

Compound	*m/z*																	
	125	139	152	153	182	196	209	210	221	235	238	251	252	266	277	294	308	350
Monomethyluric acids (M_r 182)																		
1MU		2	3		44[a]	9			6		39[b]	10			9	100[c]		44[d]
3 MU		12	4		64[a]	21			26		72[b]	15			57	100[c]		55[d]
7MU		15	30		64[a]	73			33		39[b]	9			83	100[c]		58[d]
Dimethyluric acids (M_r 196)																		
1,3MU		17	2	2		100[a]	15			6			60[b]				25[c]	
1,7MU		1	2	19		100[a]	8			60			63[b]				55[c]	
3,7MU		23	28	6		100[a]	13			6			57[b]				16[c]	
Trimethyluric acid (M_r 210)																		
TMU	7			34				100[a]						25[b]				

[a] Methyluric acid ions.
[b] Monobutylmethyluric acid ions
[c] Dibutylmethyluric acid ions
[d] Tributylmethyluric acid ions.

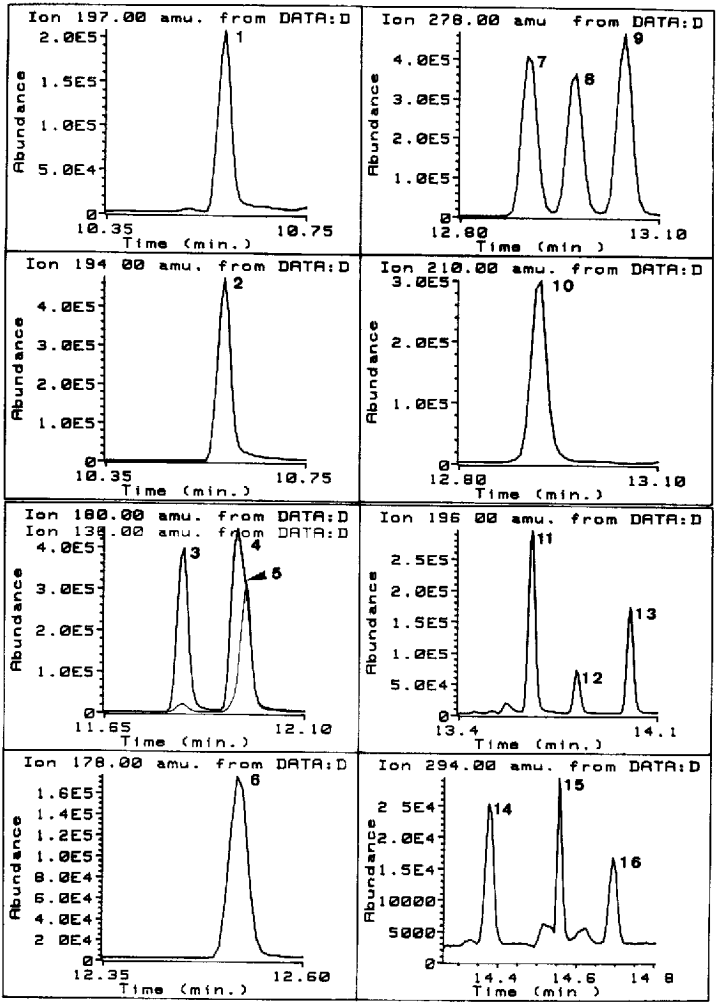

Fig. 1. Fragmentogram of the extract of a hepatocyte-free medium spiked with (1) CAF*, (2) CAF, (3) 1,3MX, (4) and (5) 3,7MX and 1,7MX, (6) IBMX, (7) 3MX, (8) 1MX, (9) 7MX, (10) TMU, (11) 1,3MU, (12) 1,7MU, (13) 3,7MU, (14) 1MU, (15) 3MU and (16) 7MU (1 mg/l each)

relative retention times, with sample standard deviations (S.D.) and coefficients of variation (C.V.) measured on 50 separate chromatographic runs.

Two pairs of compounds cannot be resolved: (i) 3MX and TMU: their specific fragment ions, *m/z* 278 and 210, respectively, allow individual measurements from the common chromatographic peak; (ii) 1,7MX and 3,7MX: these two compounds generate the same fragment ions at *m/z* 136 and 180. The relative abundances of these ions are quite different depending on the parent molecule. The relative intensity of the ion at *m/z* 136 is 10% in the 3,7MX mass spectrum

TABLE IV

REPRODUCIBILITY OF RETENTION TIMES

Compound	*m/z*	*n*	Retention time (min)			Relative retention time[a]		
			Mean	S.D	C.V (%)	Mean	S D.	C V (%)
CAF	194	50	10.530	0 023	0 22	1.000 07	0 000 14	0.014
CAF*	197	50	10.531	0 017	0 16	1.000 00		
1,3MX	180	50	11.821	0 011	0.09	0.944 65	0 000 15	0 016
3,7MX	180	50	11 948	0.018	0 15	0.954 84	0 000 68	0 072
1,7MX	136	50	11.963	0 012	0.10	0.956 00	0.000 19	0 020
IBMX	178	50	12 513	0 013	0.10	1.000 00		
3MX	278	50	12 902	0 011	0.09	1 031 09	0 000 21	0 021
TMU	210	50	12 916	0.012	0 09	1 032 21	0 000 17	0 017
1MX	278	50	12.970	0.011	0 09	1 036 46	0.000 21	0 021
7MX	278	50	13 045	0.011	0 08	1.042 47	0 000 24	0.023
1,3MU	196	50	13 650	0.012	0 09	1 090 86	0 000 15	0.014
1,7MU	196	50	13.810	0 013	0 09	1 103 60	0.000 14	0.013
3,7MU	196	50	13 997	0 013	0 09	1.118 61	0.000 14	0.012
1MU	294	50	14 372	0 012	0.09	1 148 57	0.000 21	0 018
3MU	294	50	14.555	0 016	0 11	1 163 19	0.000 45	0 039
7MU	294	50	14 690	0 015	0 10	1 173 92	0.000 17	0 014

[a] CAF and metabolites relative retention times are calculated *versus* CAF* and IBMX.

and 100% for its 1,7-isomer. Owing to such a difference, the simultaneous monitoring of the two ions and the use of the calibration equation described below allow the measurement of the two molecules simultaneously.

Calibration curves

The linearity of the calibration curves was tested in the following concentration ranges 0.04–20 mg/l for caffeine, 0.02–10 mg/l for MX compounds and 0.1-10 mg/l for MU derivatives. Internal standards used were CAF* for caffeine and IBMX for all other analytes. A linear regression analysis of peak-area ratios *versus* concentration gave the following results:

CAF: slope = 0.699; intercept = 0.0716; r^2 = 1.000
1,3MX: slope = 0.765; intercept = 0.0243; r^2 = 0.999
1MX: slope = 0 727; intercept = 0.0990; r^2 = 0.999
3MX: slope = 0.748; intercept = 0.0759; r^2 = 0.999
7MX: slope = 0.840; intercept = 0.0745; r^2 = 0.999
TMU: slope = 0.696; intercept = 0.0217; r^2 = 0.999
1,3MU: slope = 0.511; intercept = 0.0185; r^2 = 0.999
1,7MU: slope = 0.174; intercept = 0.0122; r^2 = 0.999
3,7MU: slope = 0.375; intercept = 0.0007; r^2 = 0.999

1MU: slope = 0.049; intercept = 0.0005; r^2 = 0.998
3MU: slope = 0.068; intercept = 0 0005; r^2 = 0.998
7MU: slope = 0.031; intercept = 0.0004; r^2 = 0.998

A two-step approach is used for quantitation of 1,7MX and 3,7MX mixtures.
(i) Determination of calibration parameters and response coefficients for each individual isomer by measuring ions at m/z 136 and 180:

Ion at m/z = 180 from 3,7MX: slope = 0.920, intercept = 0.0132; r^2 = 1.000
Ion at m/z = 136 from 3,7MX: slope = 0.078; intercept = 0.0014; r^2 = 0.999
Ion at m/z = 180 from 1,7MX: slope = 0.712; intercept = 0.0360, r^2 = 1.000
Ion at m/z = 136 from 1,7MX. slope = 0.851; intercept = 0.0072; r^2 = 1.000.

(ii) Under these conditions the concentration of each isomer in an unknown sample can be obtained by simultaneous measurement of the both ions and calculated according to:

3,7MX concentration = IBMX concentration × $[(0.851 \times A_{180} - 0.712 \times A_{136})/A_{178}]/0.727$
1,7MX concentration = IBMX concentration × $[(0.920 \times A_{136} - 0.078 \times A_{180})A_{178}]/0.727$

where A_{136}, A_{180} and A_{178} stand for the areas of the chromatographic peaks corresponding to ions at m/z 136, 180 and 178, respectively. The value of 0.727 was calculated from the slopes of individual 1,7MX and 3,7MX calibration curves: $0.727 = (0.920 \times 0.851) - (0.078 \times 0.712)$.

Accuracy and precision

The recovery, accuracy and reproducibility of the method over a one-week period (Tables V and VI) were evaluated by measuring concentrations from the five spiked standard solutions. The lowest C.V was observed for caffeine, which may be attributed to the use of its ^{13}C–^{15}N isotopomer as internal standard. MU measurements were associated with a higher C.V. (4–7.6%) than MX measurements. It may be explained by greater variations in sample treatment (extraction and derivatization) for MU, which are partially compensated, in the case of MX determination, by the use of a methylxanthine analogue (IBMX) as internal standard. In order to increase the precision, various MU analogues were tested as internal standards, but they could not be used because of very low derivatization yields (1,9MU and 7,9MU) or excessive retention time (3,9MU).

For all the measured molecules, the recovery was acceptable and the accuracy quite suitable for routine analysis (99 2–101.9%), although a slight underestimation was observed with low concentrations of 3,7MX and 1,7MX

TABLE V

ACCURACY, PRECISION AND RECOVERY OF METHYLXANTHINES

Compound	Standard No.	Spiked concentration (mg/l)	*n*	Measured concentration (mean ± S D) (mg/l)	Precision (C V) (%)	Accuracy (%)		Recovery[b] (mean ± S D) (%)
						A[a]	Mean ± S D	
CAF	1 + 2	0.5	10	0 505 ± 0 010	2 0	100 96	100.23 ± 0 773	104.7 ± 2 9
	3	3	5	2.975 ± 0.034	1 1	99 16		
	4 + 5	6	10	6.034 ± 0 048	0 8	100 57		
1,3MX	1 + 2	0.25	10	0.243 ± 0.005	2.2	97 38	99 20 ± 1 310	88 2 ± 3 4
	3	1.5	5	1.506 ± 0.054	3.6	100 40		
	4 + 5	3	10	2.995 ± 0.043	1.4	99.83		
1,7MX	1	0.25	5	0.209 ± 0.006	3.1	83.57	96.33 ± 8 638	92 0 ± 3 8
	4	0.25	5	0.235 ± 0.009	3.6	94.01		
	3	1.5	5	1.654 ± 0 117	7.0	110.27		
	2	3	5	2.789 ± 0.192	6.9	92.98		
	5	3	5	3.164 ± 0.094	2.9	105.46		
3,7MX	1	0.25	5	0.243 ± 0.005	2 2	97.00	96.54 ± 8.215	85.3 ± 3.6
	2	0.25	5	0.205 ± 0.011	5 4	81 84		
	3	1 5	5	1 645 ± 0 069	4.2	109.70		
	4	3	5	2.911 ± 0.108	3.7	97 03		
	5	3	5	3 045 ± 0.097	3 2	101 50		
1MX	1 + 2	0 25	10	0 245 ± 0.008	3 2	98 18	99 62 ± 1 025	101.6 ± 6.6
	3	1 5	5	1 507 ± 0.053	3 5	100.48		
	4 + 5	3	10	3 006 ± 0 048	1 6	100.20		
3MX	1 + 2	0 25	10	0 252 ± 0.008	3 0	100.98	100 49 ± 0.358	100 1 ± 4 2
	3	1 5	5	1 505 ± 0 044	2 9	100.34		
	4 + 5	3	10	3 004 ± 0 093	3 1	100.14		
7MX	1 + 2	0 25	10	0 253 ± 0 008	3 1	101.10	100 53 ± 0 429	97.5 ± 2 1
	3	1 5	5	1 506 ± 0 038	2 5	100 41		
	4 + 5	3	10	3 002 ± 0 087	2 9	100 07		

[a] Accuracy is calculated as $100 - (100dx/x)$.

[b] $n = 4$

TABLE VI

ACCURACY, PRECISION AND RECOVERY OF METHYLURIC ACIDS

Compound	Standard No	Spiked concentration (mg/l)	n	Measured concentration (mean ± S.D.) (mg/l)	Precision (C.V.) (%)	Accuracy (%)		Recovery[b] (mean ± S.D.) (%)
						A[a]	Mean ± S.D.	
TMU	1 + 2	0.25	10	0.248 ± 0.014	5.8	99.39	100.15 ± 0.727	107.7 ± 9.8
	3	1.5	5	1.502 ± 0.085	5.7	101.13		
	4 + 5	3	10	2.998 ± 0.161	5.4	99.93		
1,3MU	1 + 2	0.25	10	0.263 ± 0.020	7.6	105.21	100.86 ± 2.383	74.8 ± 7.4
	3	1.5	5	1.498 ± 0.091	6.1	99.87		
	4 + 5	3	10	3.015 ± 0.219	7.3	100.50		
1,7MU	1 + 2	0.25	10	0.257 ± 0.012	4.7	102.81	100.94 ± 1.326	101.3 ± 12.8
	3	1.5	5	1.500 ± 0.067	4.5	100.07		
	4 + 5	3	10	2.998 ± 0.135	4.5	99.93		
3,7MU	1 + 2	0.25	10	0.250 ± 0.014	5.6	100.01	99.98 ± 0.299	88.2 ± 8.9
	3	1.5	5	1.494 ± 0.066	4.4	99.60		
	4 + 5	3	10	3.010 ± 0.165	5.5	100.33		
1MU	1 + 2	0.25	10	0.250 ± 0.020	7.2	100.17	99.72 ± 0.450	63.6 ± 8.3
	3	1.5	5	1.489 ± 0.081	5.5	99.27		
	4 + 5	3	10	3.008 ± 0.251	7.2	100.27		
3MU	1 + 2	0.25	10	0.251 ± 0.013	5.3	100.41	100.24 ± 0.170	36.5 ± 3.5
	3	1.5	5	1.501 ± 0.068	4.5	100.07		
	4 + 5	3	10	3.008 ± 0.156	5.2	100.28		
7MU	1 + 2	0.25	10	0.253 ± 0.018	7.2	101.21	100.81 ± 0.405	52.4 ± 4.9
	3	1.5	5	1.506 ± 0.087	5.8	100.40		
	4 + 5	3	10	3.009 ± 0.120	4.0	100.30		

[a] Accuracy is calculated as $100 - (100 dx/x)$

[b] $n = 4$.

Application of assay

This method was applied to examine the ability of freshly isolated hepatocytes to metabolize caffeine. Hepatocytes were isolated from fed adult male Wistar rats (250–350 g) using the collagenase perfusion technique of Berry and Friend [18] as modified by Riou *et al* [19]. Cell viability was controlled, throughout the incubation period (90 min) by the trypan blue exclusion test (>90% at t_0), the measurement of ATP content (*ca.* 2 μmol/g) and the ratio of extracellular to total

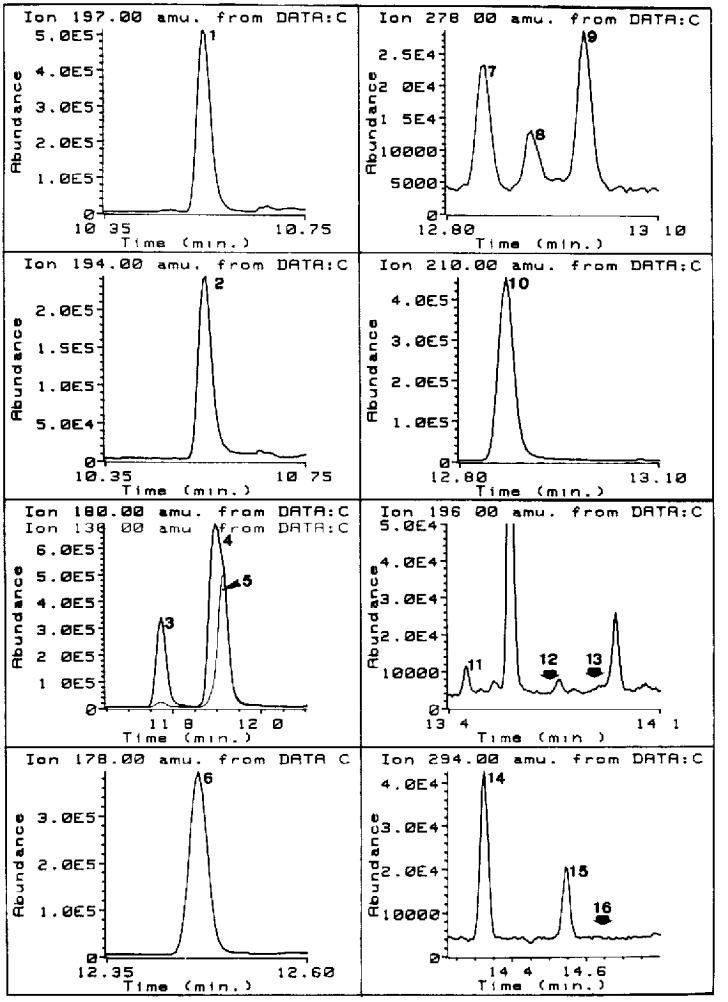

Fig 2. Fragmentogram of the extract of an extracellular medium obtained from isolated rat hepatocytes (2 3 · 10^6 cells per ml) pretreated with 3-methylcholanthrene and incubated with caffeine (14 3 μmol per 10^6 cells) over a 90-min period· (1) CAF*; (2) CAF; (3) 1,3MX; (4) and (5) 3,7MX and 1,7MX, (6) IBMX, (7) 3MX; (8) 1MX, (9) 7MX, (10) TMU, (11) 1,3MU, (12) 1,7MU, (13) 3,7MU, (14) 1MU; (15) 3MU and (16) 7MU

LDH. The cell concentration was set at 2–2.5 · 10^6 cells per ml, and the concentration of caffeine was *ca.* 35 μM. Without co-carcinogen induction, caffeine metabolism, in this model, was found to be negligible. Optimal induction conditions were obtained with a single dose of 3-methylcholanthrene (30 mg/kg, intraperitoneally), 24 h before hepatocyte isolation. Under these conditions, the apparent half-life of caffeine (56 ± 20 min, $n = 6$) indicated a great variability in the metabolic capacity of the suspensions of hepatocytes. Moreover, a gradual formation of seven caffeine metabolites could be observed: as with *in vivo* studies [10], the first to be formed were 1,7MX, 3,7MX, 1,3MU and TMU. Between 30 and 60 min, 1,3MU, 1MU, 1MX and 3MX appeared in the medium. At the end of the incubation period (90 min), the recovery of caffeine and measured metabolites ranged from 79 to 97% of the initial caffeine concentration (mean ± S.D.: 89 ± 5%; $n = 6$). This indicates the presence of other caffeine metabolites in the medium, as reported previously [10,20]. Finally, caffeine uptake by hepatocytes was found very reproducible during the six experiments (34 ± 4%).

Fig. 2 shows the fragmentogram corresponding to the assay of an extracellular medium after caffeine incubation (14.3 μmol per 10^6 cells) during 90 min. The concentrations (μmol per 10^6 cells) of the various metabolites are: CAF, 1 50; 1,3MX, 1.43; 3,7MX, 1.93; 1,7MX, 1.98; 3MX, 0.094; 1MX, 0.052; 7MX, 0.11; TMU, 2.17; 1,3MU, 0.51 and 1MU, 2.53 (1,7MU, 3,7MU and 7MU were below the detection limit.)

DISCUSSION

Three types of consideration motivated our preference for the classical liquid–liquid extraction technique using a binary solvent. (i) Direct injection following biological sample deproteinization gives rise to numerous interfering peaks, rapid fouling of the injector and premature column deterioration. (ii) Although it is practical [21–24], solid-phase extraction remains rather expensive for everyday practice. Moreover, the use of anion-exchange supports is particularly time-consuming [25,26] because the elution parameters of MU and MX are quite different. (iii) Ion-pair liquid extraction, although reported as permitting quantitative recovery of MMX and MU [27,28], gave poor results because of the very low derivatization yields. In order to optimize the conditions for simultaneous MU and MX extraction, three factors were examined. After tests in the range 0–50%, the proportion of 2-propanol was set at 15%; a higher percentage increased the extraction of polar substances that can interfere in the assay. The pH and salting-out effects were tested using ammonium sulphate (0.5 g/ml) in the extraction medium and pH values of 1, 3, 6 and 11. MX recovery was relatively constant in acidic medium and varied inversely with polarity (DMX; 75%; MMX, 40–68%), it fell off dramatically at pH 11 (20 to 1%, respectively). Addition of ammonium sulphate improved the extraction (DMX, 85%; MMX, 79–91%). Caffeine extraction was unaffected by these changes in conditions. MU extraction yields de-

creased according to their intrinsic polarity and the pH of the medium, whether the aqueous phase had been saturated or not.

Derivatization of caffeine metabolites for GC analysis has been described mainly for theophylline [29,30], less for other MX [31–34] and scarcely at all for MU [34,35]. Three major techniques have been reported: extractive alkylation using alkyl iodides and quaternary ammonium ions [32], flash alkylation [31] or alkylation using iodoalkane in polar organic solvents such as N,N-dimethylacetamide or N,N-dimethylformamide in the presence of strong mineral [34] or organic [33] bases. Extractive alkylation using high concentrations of tetramethylammonium ion in aqueous solution and iodopropane in chloroform failed to recover polar metabolites [34]. Flash alkylation was associated with poor reproducibility. Consequently, alkylation according to the Greeley's procedure was used.

CONCLUSION

The method developed was suitable for the measurement of the concentrations of all MX and MU metabolites of methylxanthines, especially caffeine, from hepatocyte incubation medium used for metabolic studies. Intracellular as well as extracellular fractions can be analysed by such a method. The analytical parameters of the technique were sufficient for an accurate estimation of metabolic kinetic parameters during *in vitro* studies. Owing to the monitoring of characteristic ions and to the high reproducibility of relative retention times, GC–MS showed a better specificity than any other chromatographic method. This is particularly relevant in order to discriminate peaks of low intensity that correspond to metabolites from potentially interfering compounds. Moreover, such a methodology affords a suitable analytical tool when using the cluster technique with mixtures of molecules labelled with stable isotopes and unlabelled analogues for metabolic investigations, as we applied it for a study of the metabolism of seven deuterated analogues of caffeine [15,36].

REFERENCES

1 J J. Barone and H Roberts, in P B. Dews (Editor), *Caffeine, Perspectives from Recent Research*, Springer-Verlag, Berlin, 1984, pp 59–73.
2 K Hirsh, in G A Spiller (Editor), *Progress in Clinical and Biological Research*, Vol. 158, Alan R Liss, New York, 1984, pp 235–302
3 B. B Fredholm, in G A Spiller (Editor), *Progress in Clinical and Biological Research*, Vol 158, Alan R. Liss, New York 1984, pp. 303–330.
4 A. B. Becker, K J Simons, C A Gillespie and F E R. Simons, *N Engl J Med.*, 310 (1984) 743.
5 E Renner, H Wietholtz, H. Huguenin, M. J Arnaud and R Preisig, *Hepatology*, 4 (1984) 38
6 R. Joeres, H. Klinker, H. Heusler, J. Epping, G. Hofstetter, D Drost, H. Reuss, W Zilly and E Richter, *Arch Toxicol*, 60 (1987) 93
7 N R Scott, D. Stambuk, J Chakraborty, V Marks and M Y. Morgan, *Clin. Sci*, 74 (1988) 377
8 D D-S Tang-Liu, R L Williams and S Riegelman, *J Pharmacol. Exp. Ther.*, 224 (1983) 180
9 M. J. Arnaud, in P. B. Dews (Editor), *Caffeine, Perspectives from Recent Research*, Springer-Verlag, Berlin, 1984, pp 3–38

10 M Bonati, R Latini, G Tognoni, J. F. Young and S. Garattini, *Drug Metab. Rev.*, 15 (1984-1985) 1355.
11 R. Gorodischer, A. Yaari, A Margalith, D Warzawski and Z Ben-zvi, *Biochem Pharmacol.*, 35 (1986) 3077.
12 M E Campbell, D M. Grant, T. Inaba and W. Kalow, *Drug Metab. Dispos*, 15 (1987) 237.
13 F. Berthou, D Ratanasavanh, D Alix, D Carlhant, C Riche and A Guillouzo, *Biochem Pharmacol.*, 37 (1988) 3691.
14 M E McManus, J O Miners, D Gregor, I. Stupans and D J Birkett, *J Pharm. Pharmacol.*, 40 (1988) 38.
15 B. Ribon, Y. Benchekroun, M. Desage and J L. Brazier, in A Guillouzo (Editor), *Liver Cells and Drugs, Colloque INSERM*, Vol 164, John Libbey Eurotext, London, Paris, 1988 pp 269–274
16 R. H. Greeley, *J Chromatogr*, 88 (1974) 229
17 M Desage, J Soubeyrand, A. G Soun, J. L. Brazier and Y George, *J. Chromatogr*, 336 (1984) 285.
18 M. N. Berry and D. J Friend, *J Cell Biol.*, 43 (1969) 506
19 J P Riou, T. H. Claus and J. J. Pilkis, *Biochem. Biophys. Res. Commun.*, 73 (1976) 591
20 F Berthou, D. Ratanasavanh, C. Riche, D. Picart, T. Voirin and A. Guillouzo, *Xenobiotica*, 19 (1989) 401.
21 R. Hartley, I J Smith and J R. Cookman, *J. Chromatogr.*, 342 (1985) 105.
22 M B Kester, C L Saccar, M L Rocci and H. C. Mansmann, *J. Pharm Sci*, 76 (1987) 238.
23 E. Naline, B. Flouvat, C Advenier and M Pays, *J Chromatogr*, 419 (1987) 177.
24 K Matsumoto, H Kikuchi, H. Iri, H. Takahasi and M. Umino, *J. Chromatogr.*, 425 (1988) 323
25 A W Burg and M. E. Stein, *Biochem Pharmacol*, 21 (1972) 909.
26 R. D. Thompson, H. T Nagasawa and J W Jenne, *J Lab Clin. Med*, 84 (1974) 585
27 D D-S. Tang-Liu and S Riegelman, *J. Chromatogr. Sci.*, 20 (1982) 155.
28 N R Scott, J Chakraborty and V Marks, *J. Chromatogr.*, 375 (1986) 321.
29 D J F Rowe, I. D. Watson, J Williams and D. Berry, *Ann. Clin Biochem*, 25 (1988) 4.
30 D. R. Knapp (Editor), *Handbook of Analytical Derivatization Reactions*, Wiley, New York, 1979
31 M. Kowblansky, B M Scheinthal, G. D. Cravello and L. Chafetz, *J. Chromatogr*, 76 (1973) 467
32 S. Floberg, B Lindstrom and G Lonnerholm, *J Chromatogr.*, 221 (1980) 166.
33 E Houghton, *Biomed Mass Spectrom.*, 9 (1982) 103.
34 K. Y Tserng, *J. Pharm Sci*, 72 (1983) 526.
35 A H Van Gennip, J Grift, E. J. Van Bree-Blom, D. Ketting and S. K Wadman, *J. Chromatogr*, 163 (1979) 351.
36 Y Benchekroun, *Ph.D. Thesis 179.89*, University Claude Bernard, Lyon I, Lyon, 1989