Pergamon

Detection of ^{13}C labelled compounds by gas chromatography coupled to atomic emission detection — application to caffeine metabolites

M.S. BOUKRAA, D. DERUAZ,* A. BANNIER, M. DESAGE and J.L. BRAZIER

Laboratoire d'Etudes Analytiques et Cinétiques du Médicament — LEACM, Institute of Pharmaceutical and Biological Sciences, 8 avenue Rockefeller, 69373 Lyon cedex 08, Lyon, France

Abstract: This paper illustrates the use of gas chromatography coupled to atomic emission detection (GC–AED) and of the stable isotope tracer ^{13}C for the determination of drug metabolites. After administration of a parent drug labelled with ^{13}C and extraction of the metabolites from the biological fluids, a ^{13}C chromatographic profile is determined using the specific detection of the ^{13}C atomic emission and subtraction of the ^{13}C natural abundance. Thus, only the compounds which are metabolites with a ^{13}C enrichment over the natural abundance are detected. [1,3,7 trimethyl-^{13}C$_3$]xanthine which is extensively metabolized by the liver is used as an example.

Keywords: *Atomic emission detection; microwave-induced plasma; mass spectrometry; isotopes of carbon; caffeine metabolites; methylxanthines; methyluric acids.*

Introduction

There is an increasing need in pharmaceutical and biomedical analysis for efficient separation techniques like capillary gas chromatography to be coupled with specific detectors. The interest of coupling such specific modes of detection with highly resolutive techniques increases with the development of various kinds of chromatographic systems coupled with organic mass spectrometry (MS), isotope ratio mass spectrometry (IRMS) and atomic emission detection (AED). The main features of the specific atomic emission detector are the possible simultaneous detection of several elements and isotopes and the very high level of specificity. With such a detector, it is also possible to calculate inter-element ratios and to determine the elemental composition of compounds eluted from a GC-capillary column.

AED allows the simultaneous detection of various elements and isotopes, and can replace several detection devices such as flame ionization, electron capture, thermionic and flame photometric detectors. With such a detector, the simultaneous and specific detection of hydrogen and its stable isotope, deuterium, was performed in 1973 by McLean *et al.* [1]. These works have been developed by Thomas *et al.* [2], Deruaz [3] and Bannier [4]. Other

elements and their stable isotopes were also studied, such as ^{12}C and ^{13}C [5], especially for the use of this heavy isotope of carbon as a stable tracer in metabolic studies performed in humans.

The aim of this paper is to show how such a detector can be used for the determination of drug metabolites after administration of a parent drug labelled with the stable isotope; ^{13}C. The use of stable isotopically labelled (SIL) drugs for the studies of metabolic pathways in humans increases from year to year. These SIL molecules act as safe and non-radioactive tracers. They can be used according to the 'ion cluster' technique where a mixture of unlabelled and SIL parent drug is administered to a subject. Thus, all the metabolites which are formed from this mixture are also labelled with the same isotopic content as the parent drug. They can be easily detected from the total ion current, by the presence of ion clusters corresponding to unlabelled and labelled ions. The SIL parent drug can also be administered alone and its labelled metabolites specifically detected by atomic emission detection. We report the study of the urinary metabolites of caffeine (1,3,7-trimethylxanthine) labelled with three atoms of ^{13}C on the three methyl groups located at the N1, N3 and N7 positions. Caffeine is heavily consumed

---

* Author to whom correspondence should be addressed.

in beverages [6] and is used as a central nervous system stimulant. Caffeine is also used as a metabolic probe for exploring oxidative metabolic pathways (cyt P450 IIA2) and the numerous factors that can potentially alter these enzymatic activities [7]. Demethylation mediated by microsomal monooxygenases and oxidative reactions on the C8 position, lead to the production of di- and monomethyl-xanthines (MX), tri-, di- and monomethyl uric acids (MU) as well as ring-opened uracil related metabolites [8]. With such an extensive metabolism, more than 85% of the dose of caffeine administered to a human is eliminated as urinary metabolites. If caffeine is labelled with ^{13}C on the three methyl groups, all the methylated metabolites which are eliminated are also labelled and can be detected from the biological fluids by the specific signal of ^{13}C.

Numerous analytical systems have been developed to measure caffeine and its metabolites. Among them, gas chromatography is widely used with various kinds of detectors: flame ionization [9], thermionic detection [10] or organic mass spectrometry [8, 11–15]. In the last few years, the atomic emission detector coupled to gas chromatography has been developed [16–19]. In this paper, it is shown how AED can be used for the rapid detection of the urinary metabolites of labelled caffeine and compare this atomic detection to gas chromatography coupled to mass spectrometry.

Materials and Methods

Equipment

Gas chromatograph coupled with atomic emission detector. A GC–AED (Hewlett–Packard HP 5921) was used. This system consisted of a HP 5890 Series II standard gas chromatograph equipped with a HP 7673A auto sampler and a HP 5895 A Chemstation.

The capillary column used was a cross-linked phenyl methyl silicone column OV1701 (50 m × 0.25 mm i.d.) the thickness of the stationary phase was 0.2 μm. The injection port temperature was set at 260°C and the transfer line at 270°C. The oven temperature programming was as follows: 110°C for 0.8 min, an increase to 180°C at 20°C min^{-1}, and then to 270°C at 5°C min^{-1}. The temperature was kept constant for 20 min. The injection was performed in the splitless mode (40s), the injected volume was

2 μl. Pure helium (99.9999%) was used as carrier gas with a head pressure of 180 kPa.

The geometry of the atomic emission detector cavity (a Beenakker cavity) enables a standing microwave (2.75 GHz), with energy focused in the centre of a quartz discharge tube to be established. The choice of the plasma gas (helium 99.9999%) and reagent gas (H_2 and O_2 under a pressure of 1 bar) and their flow rates were adjusted in order to optimize to the yield of atomic emission of the selected elements. The emission lines monitored were: 348.424 nm for nitrogen, 342.574 nm for ^{12}C and 341.712 nm for ^{13}C. The overall flow rate in the detector was 32 ml min^{-1}.

The detection was carried out with a movable photodiode array (PDA) which enables the collection of photons in a range of 50 nm within the overall interval between 160 and 800 nm [17]. The two isotopes of carbon can be detected using the emission of CO molecular band formed inside the plasma cavity using O_2 and H_2 as reactant gas. The first order lines emitted by the ^{12}CO and ^{13}CO molecular bands are at 171.28 and 170.86 nm, respectively, so the wavelength difference is very small ($\delta\lambda = 0.43$ nm). The second order lines ($\lambda^{12}CO = 342.574$ nm and $\lambda^{13}CO = 341.712$ nm) are preferred to the first order one because of a larger $\delta\lambda$ (0.86 nm). Nevertheless the resolution must be improved by an algorithm developed by Quimby *et al.* [5] to allow the simultaneous selective determination of ^{12}C and ^{13}C and the measurement of both isotopes.

Gas chromatograph coupled with mass spectrometry. The same chromatographic equipment and conditions were used for mass spectrometric detection. A Hewlett–Packard mass-selective detector (MSD-HP 5970 A) was used for the detection. The ionization was achieved by electron impact (70 eV), the electron multiplier voltage was set at 1800 V. The m/z values of the ions monitored in order to detect all the metabolites of caffeine are given in Table 1.

Chemical and reagents

Labelled caffeine ([1,3,7 trimethyl-$^{13}C_3$]xanthine) was purchased from Tracer Technologies (Somerville, MA, USA). Caffeine (1,3,7 trimethylxanthine: CAF); theophylline (1,3 dimethylxanthine: 1,3MX); paraxanthine (1,7 dimethylxanthine: 1,7MX);

Table 1
Monitoring conditions

| Peaks* | Compound | Mass spectrometric detector | | | Atomic emission detector | |
		m/z	Retention time (min)	RRT†	Retention time (min)	RRT†
1	CAF	194	14.656	1.000	16.626	1.000
2	1,3MX	180	17.527	1.196	21.054	1.266
3	1,7MX	180, 136	17.803	1.215	21.171	1.273
3'	3,7MX	180, 136	17.803	1.215	21.171	1.273
4	IBMX	236	18.826	1.000	22.343	1.000
5	TMU	210	20.254	1.076	23.701	1.061
6	3MX	306	21.582	1.146	24.966	1.117
7	1MX	306	21.785	1.157	25.204	1.128
8	7MX	306	22.065	1.172	25.401	1.137
9	1,3MU	196	24.282	1.290	27.166	1.216
10	1,7MU	196	25.110	1.334	27.736	1.241
11	3,7MU	196	26.336	1.399	28.625	1.281
12	1MU	322	30.413	1.615	31.611	1.415
13	3MU	322	31.813	1.690	32.621	1.460
14	7MU	322	33.201	1.763	33.598	1.504

*Number indicate the position of peaks in Figs 1, 3, 5, 6 and 7.
†Relative retention times are calculated versus CAF (peaks 1–3') and IBMX (peaks 4–14).

theobromine (3,7 dimethylxanthine: 3,7MX); 1 methylxanthine, 1MX; 3 methylxanthine, 3MX; 7 methylxanthine, 7MX; 3-isobutyl 1-methylxanthine, IBMX; 1,3 dimethyluric acid, 1,3MU; and 3,7 dimethyluric acid, 3,7MU were from Sigma (St Louis, MO, USA), 1 methyluric acid, 1MU; 3 methyluric acid, 3MU; 7 methyluric acid, 7MU; 1,3,7 trimethyluric acid, TMU; and 1,7 dimethyluric acid, 1,7MU were from Fluka (Buchs, Switzerland). Ammonium sulphate, toluene (99.5%), iso-propanol (99%), ethyl acetate (99%), per-chloric acid (12%) and chloroform (99%) were from Carlo Erba (Milan, Italy). N—N di-methylacetamide (99.5%), tetramethyl-ammonium hydroxide (0.1 mol l⁻¹ in isopro-panol–methanol) and iodopentane (98%) were from Fluka (Buchs, Switzerland).

Solutions. Methylxanthines were dissolved in a mixture of methanol and water (50:50, v/v). Solutions of the methyluric acids were performed in a NaOH (2 × 10⁻³ mol l⁻¹) in order to obtain concentrations of 100 mg l⁻¹.

Assay procedure

To 500 μl of urine placed in a test-tube were added 420 μl of distilled water, 600 mg of ammonium sulphate and 80 μl of perchloric acid (12%). After mixing, 10 ml of a mixture of chloroform–isopropanol (85:15, v/v) were added. The extraction was carried out by rocking the tube for 10 min. The phase separation was achieved by centrifugation

(3000g for 5 min), the organic layer was transferred to another tube and evaporated to dryness under a stream of nitrogen at 50°C. The derivatization of the free N—H groups was performed according to the technique previously described by Greeley [20]. After addition of 200 μl of N—N dimethylacet-amide and 100 μl of tetramethylammonium hydroxide to the dry residue and vigorous shaking for 10 s, 100 μl of iodopentane were added. The solution was shaken again and allowed to stand at room temperature for 12 h. Then, the solution was centrifuged in order to separate the thin tetramethylammonium iodide precipitate from the organic phase which was recovered and evaporated to dryness at 30°C. The dry residue containing the derivatized compounds was dissolved into 200 μl of toluene–ethyl acetate (5:2, v/v) for chromatographic analysis.

Results

Figure 1 shows the chromatograms obtained by gas chromatography–atomic emission detection of a derivatized extract from an aqueous solution containing caffeine and its metabolites at a concentration of 20 μg ml⁻¹ for MX and 30 μg ml⁻¹ for MU. An aliquot of this solution was submitted to the whole analytical procedure described above. Figure 1 shows the elemental chromatograms moni-tored at 342.574 nm for ¹²C (a), 341.712 nm for ¹³C (b) and 348.424 nm for N (c). As these
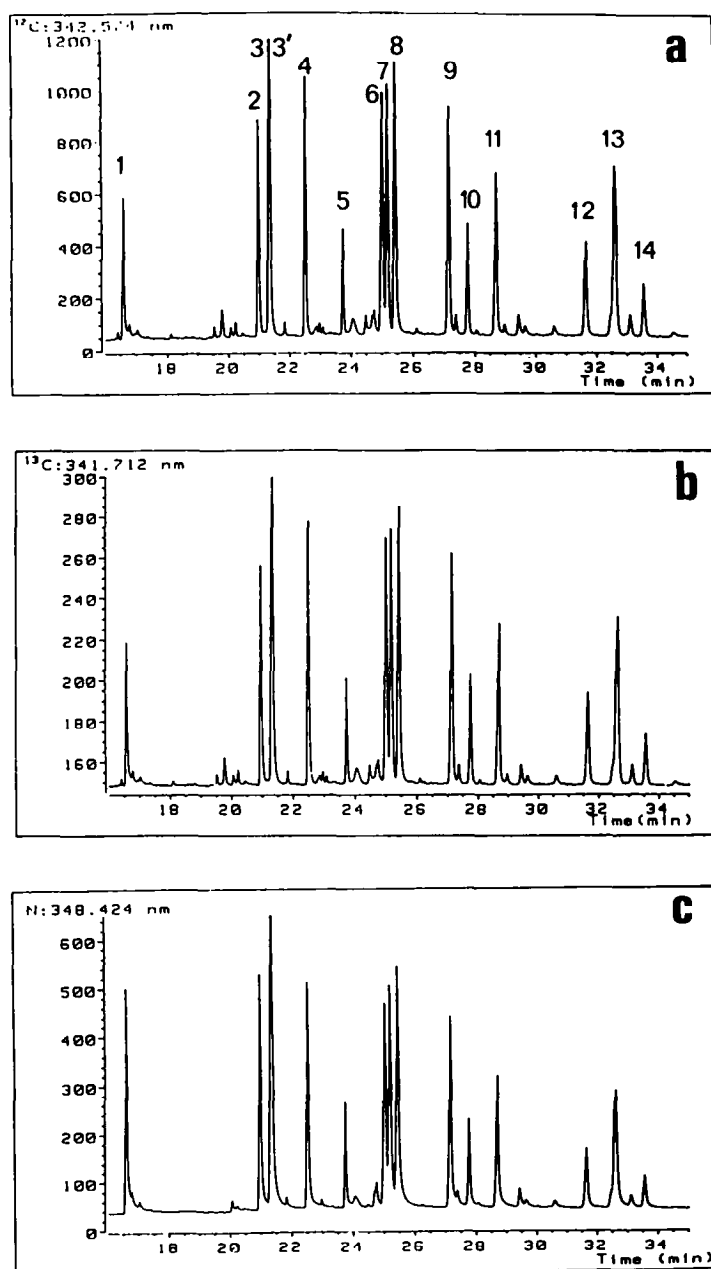
Figure 1
GC–AED: caffeine and its metabolites (peaks 1–14 in Table 1). Elemental chromatograms of ^{12}CO at 342.574 nm (a), ^{13}CO at 341.712 nm (b), N at 348.424 nm (c).

wavelengths are inside the 50 nm interval covered by the photodiode array, the three emission lines were simultaneously recorded during the same chromatographic run. When these three emission lines are used for detection, the compounds of interest which contain both stable isotopes of carbon (^{12}C and ^{13}C) and N can be detected perfectly well. Their absolute retention times as well as retention times relative to isobutylmethyl-xanthine (IBMX) are shown in Table 2.

It can be observed that the chromatogram corresponding to the detection of ^{12}C and ^{13}C are almost identical because these compounds display an isotopic abundance which is the natural ^{13}C abundance (1.1%). These results show that the AED is able to detect ^{13}C from organic compounds with an isotope enrichment corresponding to the terrestrial natural abundance of this isotope (1.01–1.15%).

Figure 2 shows the three-dimensional display of the emission lines of the ^{12}C and ^{13}C

Table 2
Mass spectra of pentylated methylxanthines: relative abundances of major peaks

Compound	m/z											
	109	122	136	166	180	192	193	194	207	236	250	306
Isobutylmethylxanthine (Mr 222)												
IBMX		36		63*		90	30		50	100†		
Monomethylxanthines (Mr 166)												
1MX		40	24	70*	44	78				100†	42	68‡
3MX				100*	63		28	22	36	90†		82‡
7MX			100	84*	85		25	20	36	57†		88‡
Dimethylxanthines (Mr 180)												
1,3MX	30				100*		29	27	58		65‡	
1,7MX			67		100*		25	25	35		44‡	
3,7MX					100*		28				49‡	
Trimethylxanthine (Mr 194)												
CAF	61							100*				

* Methylxanthine ions.
† Monopentylmethylxanthines ions.
‡ Dipentylmethylxanthine ions.
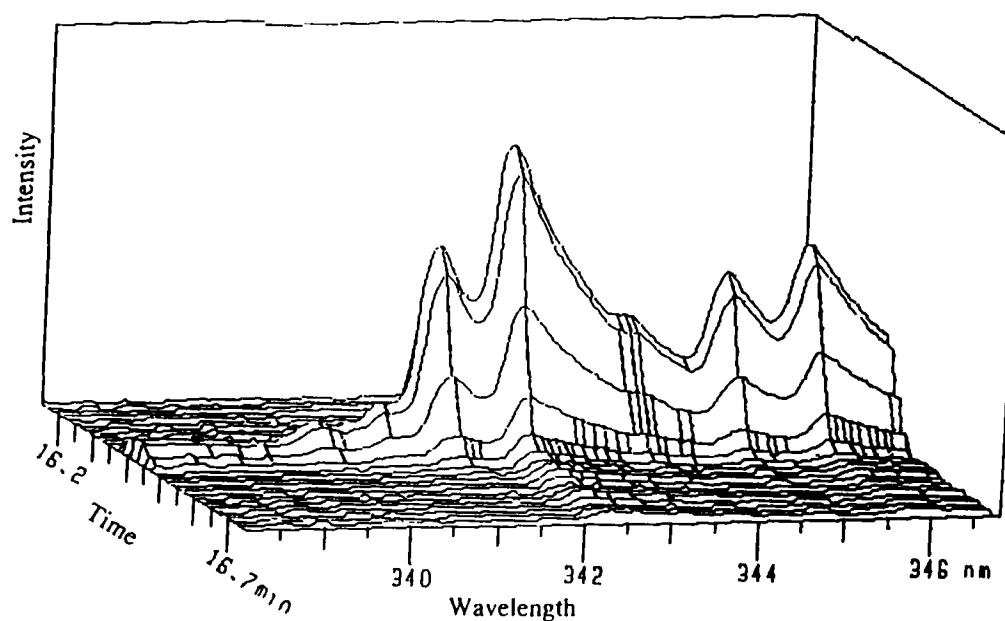
Figure 2
GC–AED: three-dimensional display of the snapshots of ¹²CO and ¹³CO emission bands recorded during the elution of the peak corresponding to [¹³C₃]caffeine between 16.2 and 16.7 min (338–346 nm).

molecular bands recorded on-line during the chromatographic run and corresponding to the [¹³C₃][¹²C₅] caffeine peak. The three recorded variables are the wavelengths (338–346 nm), the retention time (16.2–16.7 min) and the intensity of the emission lines. The figure shows that the emission spectra of ¹²CO and ¹³CO are overlapping but the respective signals of both carbon isotopes can be automatically extracted from the whole analytical response using the algorithm developed by Quimby et al. [5].

Figure 3 displays the chromatogram of the same solution containing caffeine and its metabolites obtained by GC–MS analysis in the SIM mode (selected ion monitoring). In order to obtain a specific detection the characteristic ions which are monitored must be changed according to the retention time windows where each compound is eluted (Table 1).
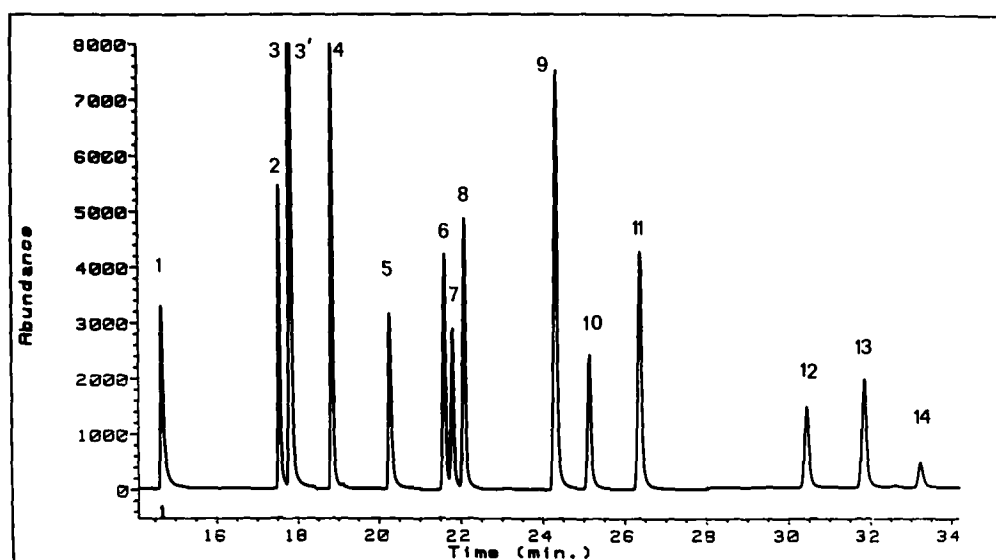
Figure 3
GC–MS: fragmentograms of caffeine and its metabolites (peaks 1–14 in Table 1): MX (20 mg l^{-1}) and MU (30 mg l^{-1}). SIM mode.

Table 3
Mass spectra of pentylated methyluric acids: relative abundances of major peaks

Compound	m/z												
	153	182	196	207	210	252	253	266	269	305	322	336	392
Monomethyluric acids (Mr 182)													
1MU				78		41†	44		72		100‡		26§
3MU		70*	33	48		86†	35		47	32	100‡		39§
7MU		53*		100			47		48	47	79‡		28§
Dimethyluric acids (Mr 196)													
1,3MU			100*					80†				21‡	
1,7MU			100*	20				97†				23‡	
3,7MU	20		100*					58†				21‡	
Trimethyluric acid (Mr 210)													
TMU	40				100*								

* Methyluric acid ions.
† Monopentylmethyluric acid ions.
‡ Depentylmethyluric acid ions.
§ Tripentylmethyluric acid ions.

Under these analytical conditions all the metabolites could be selectively detected and measured. The identity of these compounds was verified in the scan mode by the presence of their characteristic molecular and fragment-ions. The results are given in Table 2 for methylxanthines and Table 3 for methyluric acids.

A blank urine sample from a subject who was not a caffeine consumer was spiked with a solution containing caffeine labelled with ^{13}C and 12 unlabelled metabolites as well as IBMX (internal standard) in order to obtain a final concentration of 20 μg ml^{-1} for MX and 30 μg ml^{-1} for MU. An aliquot sample was processed according to the described procedure.

Figure 4 shows the elemental chromato-grams from the extracted urine where the following elements or isotopes are recorded: ^{12}C (a), ^{13}C (b) and N (c). As every compound present in urine contains both carbon isotopes, the corresponding elemental chromatograms are quite non-specific and unable to detect the presence of caffeine and its various meta-bolites. Numerous molecules excreted by urine also contain nitrogen and the N chromatogram
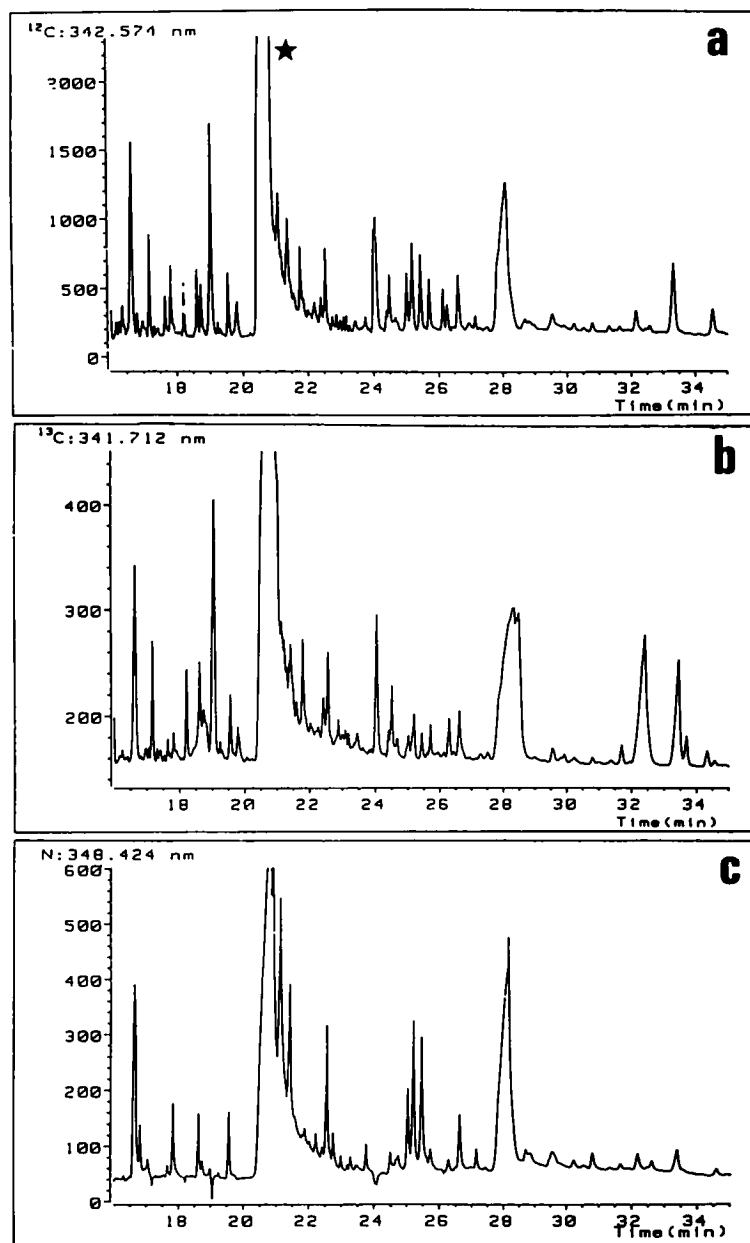
Figure 4
GC–AED: elemental chromatograms obtained from a urinary extract. Caffeine and metabolites described in Table 1.
MX, 20 mg l⁻¹; MU, 30 mg l⁻¹. (a) ^{12}CO at 342.574 nm, (b) ^{13}CO at 341.712 nm, (c) N at 348.424 nm; *: hippuric acid.

is no more specific for caffeine detection. The very large peak on the chromatogram corresponds to hippuric acid whose concentration in urine is very high.

A selective detection can be performed by GC–MS. In the SIM mode, the 14 molecules of interest can be specifically found on the condition that the monitored ion be changed for the detection of each series of compounds according to the m/z values of their characteristic ions (Fig. 5).

A specific detection can also be performed by GC–AED. The ^{13}C chromatogram in Fig. 4(b) corresponds to the analytical response given by the natural abundance of this carbon isotope. All the compounds which appear on this chromatogram have the same ^{13}C enrichment (1.1 %), caffeine excepted which is artificially enriched on the three methyl groups. This ^{13}C elemental chromatogram can be processed in order to subtract the natural abundance of ^{13}C. The signal used for this
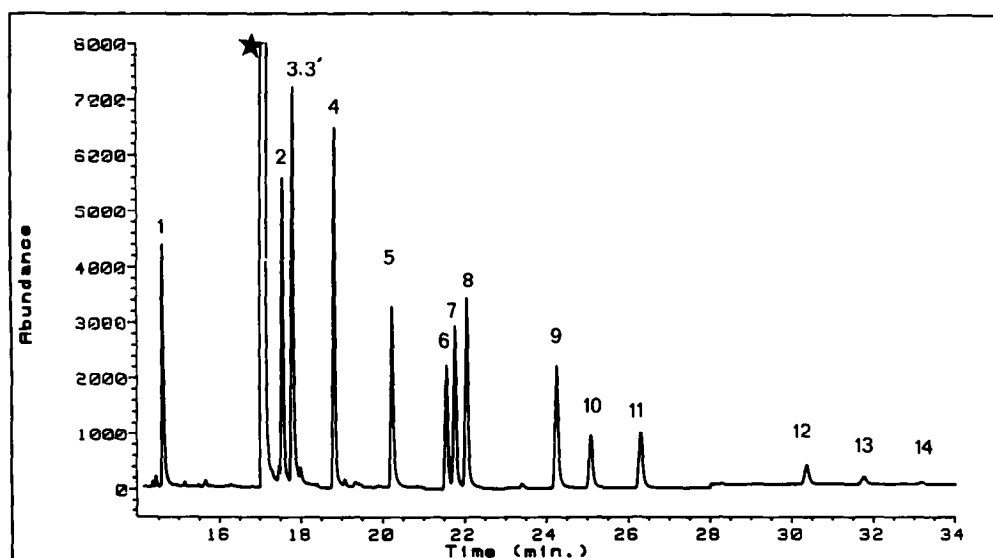
Figure 5
GC–MS: fragmentogram obtained from a urinary extract. Caffeine and metabolites described in Table 1. MX, 20 mg l⁻¹; MU, 30 mg l⁻¹; *: hippuric acid.
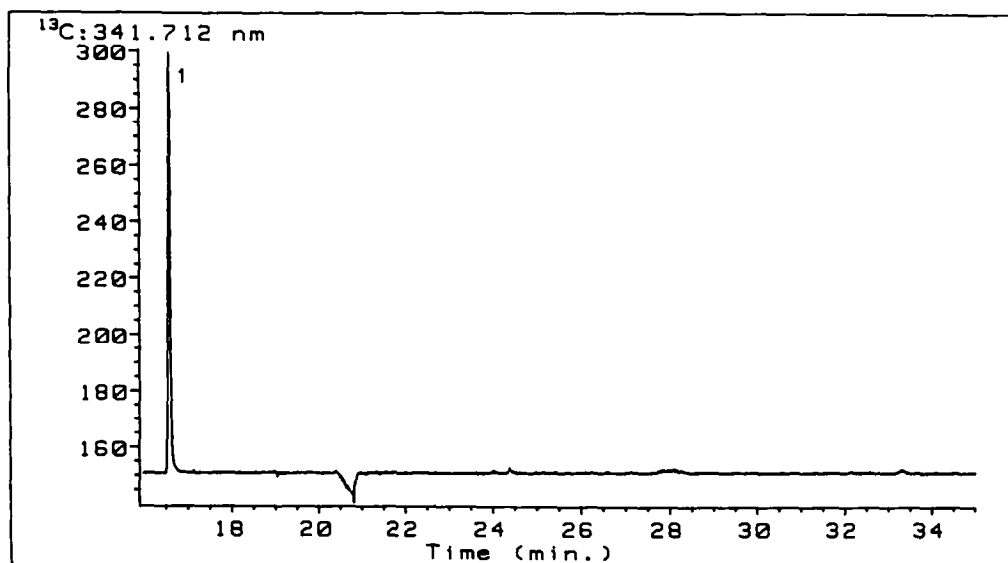

Figure 6
GC–AED: ¹³C profile of a urine sample spiked with [¹³C]caffeine after processing the data with the 'SUPPRESS' function.

subtraction is taken from a portion of the chromatogram free of peaks corresponding to labelled molecules (10–13 min). The use of this 'SUPPRESS' function results in the total removing of the natural ¹³C response. So only the compounds enriched with ¹³C over the natural abundance are specifically detected.

Figure 6 displays the results of the 'SUPPRESS' function on the ¹³C chromatogram of the spiked urine where only caffeine is

labelled with ¹³C. Only caffeine can be detected because of this artificial enrichment. Caffeine (5 ng) was injected into the chromatographic column, the corresponding amount of ¹³C is 0.99 ng. The minimum detectable limit (MLD) defined by Quimby *et al.* [5] as 'the amount of element required to produce a peak twice the height of the peak to peak noise, divided by the full width at half height of the peak in seconds' can be calcu-

lated. The width at half height of the ^{13}C caffeine peak was 6.5 s, so the sensitivity of the detection according to Quimby is 150 pg s^{-1} of ^{13}C. The area under the chromatographic peak displayed here was 1018 (arbitrary area units). The minimum area which can be usually measured in proportion to the noise is about 50 (area units) so the MDL for ^{13}C is near 7.5 pg s^{-1}.

The same method was used to find the various metabolites of caffeine from the urine of a subject who absorbed an oral dose of 50 mg of caffeine labelled with three atoms of ^{13}C. The ^{13}C enrichment of each methyl group was 99%, so the total amount of ^{13}C administered as caffeine was 9.8 mg. Figure 7(a) shows the isotopic ^{13}C chromatogram from the urine collected before caffeine intake processed to subtract the natural ^{13}C contribution.

No significant peak can be observed on the chromatogram because all the molecules extracted from urine and eluted from the chromatograpic column have the same ^{13}C content which is the natural one. Figure 7(b) shows the subtracted ^{13}C chromatogram of a urine sample collected from 6 to 9 h after labelled caffeine intake. The only peaks which can be observed on this subtracted chromatogram are those of caffeine and its urinary because these molecules are enriched in ^{13}C over the natural abundance and are not erased by the natural ^{13}C subtraction.

Conclusion

This example clearly shows that atomic emission detection coupled with gas chromatography is a powerful tool for the screening
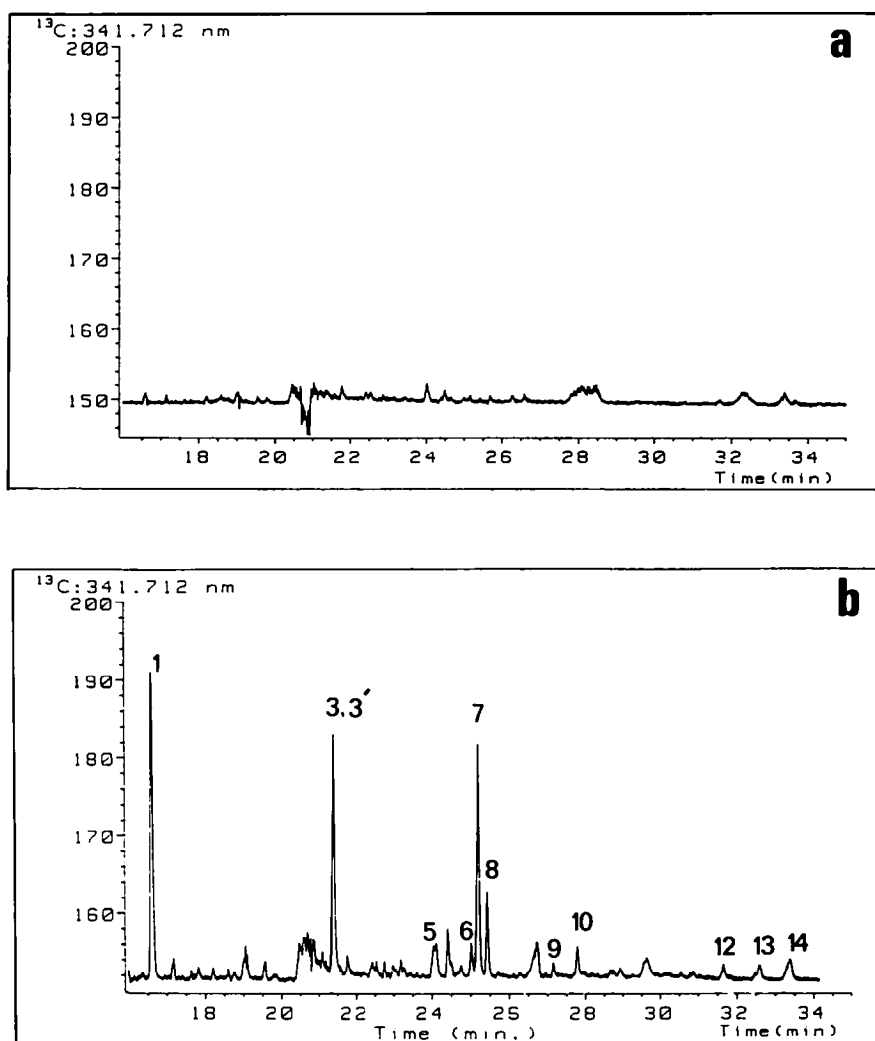

Figure 7
GC–AED profile obtained from a urine extract: (a) before caffeine intake, (b) from the urine of a subject taking [^{13}C]caffeine. These profiles are the results of data processing with the 'SUPPRESS' function.

of compounds from complex matrices and mixtures using the selective detection of an element or isotope. Despite the very small wavelength difference between the emission lines of ^{12}CO and ^{13}CO (0.85 nm for the second order), the algorithm proposed by Quimby allows the extraction of both isotopic signals and the perfect recording of the specific ^{13}C chromatogram. These second order emission lines are preferred to the first order one (at 171.3 and 170.8 nm, respectively) which are more intense but too close to one another to allow a good extraction of the ^{13}C response at the ^{13}C natural abundance level. The processing of the ^{13}C chromatogram and the judicious use of the 'SUPPRESS' function allows the subtraction of the natural contribution of ^{13}C and a specific detection of the only molecules whose ^{13}C enrichment is over the natural one. So gas chromatography coupled with atomic emission detection is a very appropriate tool for metabolic studies where the parent drug is labelled with stable isotopes (like ^{13}C). If the labelled site is correctly chosen in order not to be removed during the metabolic process, the label remains in all the metabolites formed from the parent drug. A non-specific extraction of the biological fluid to be studied, followed by a chromatographic analysis with ^{13}C atomic emission detection with the subtraction of the ^{13}C natural abundance allows the various ^{13}C enriched compounds corresponding to the drug metabolites from the whole chromatogram to be detected. In this way a specific profile of metabolites can be obtained. Another alternative to detect metabolites is GC–MS and the ion-cluster technique with co-administration of a mixture of both unlabelled and labelled parent drugs. When using such a method, ion clusters have to be detected for each unknown metabolite. An algorithm can be used to systematically seek for these clusters along the total ion current. This detection can be very difficult if the compounds are at a very low level and their corresponding ion cluster within the noise intensity. An alternative is to use the selected ion monitoring mode (as shown above). The disadvantage of this method is the necessity to know the characteristic fragment-ions of the metabolites and generally neither the metabolites nor their characteristic ions are known before the study of metabolic pathways.

Consequently, the major advantages of GC–AED for the screening of the metabolites of

labelled drugs relies on the possibility (i) of on-line monitoring various elements or isotopes and among them the tracer isotope ^{13}C during the same chromatographic run, (ii) to use and compare the elemental responses for inter-element ratios calculations, (iii) to use the subtraction of the ^{13}C natural abundance from the whole ^{13}C chromatogram in order to easily point out only the labelled compounds.

GC–AED is therefore a new powerful analytical tool which can be used for elemental and/or isotopic screening of molecules from complex mixtures. The calculation of inter-element ratios can allow the determination of the empirical formula of the eluted compound. Moreover, GC–AED analysis can be used as a screening step before a structural determination by GC–MS on the same sample. These two techniques are complementary in an analytical strategy process.

References

[1] W.R. McLean, D.L. Stanton and G.E. Penketh, *Analyst* 98, 432–442 (1973).
[2] L.C. Thomas and T.L. Ramus, *J. Chromatogr.* 586, 309–313 (1991).
[3] D. Deruaz, A. Bannier, M. Desage and J.L. Brazier, *Anal. Letts* 24, 1531–1543 (1991).
[4] A. Bannier, D. Deruaz, C. Weber and J.L. Brazier, *Anal. Letts* 25, 1073–196 (1992).
[5] B.D. Quimby, P.C. Dryden and J.J. Sullivan, *Anal. Chem.* 62, 2509–2512 (1990).
[6] J.J. Barone and H. Roberts, in *Caffeine, Perspectives from Recent Research* (P.B. Dews, Ed.), pp. 59–73. Springer-Verlag, Berlin (1984).
[7] W. Kalow, *Clin. Pharm. Therap.* 49, 44–48 (1991).
[8] Y. Benchekroun, M. Désage, B. Ribon and J.L. Brazier, *J. Chromatogr.* 532, 261–275 (1990).
[9] V.P. Shah and S. Riegelman, *J. Pharm. Sci.* 63, 1283–1285 (1974).
[10] J.F. Van de Calseyde and J. Vander Veeken, *Pharm. Weekblad Scientic. Ed.* 4, 20 (1982).
[11] J.L. Brazier, Y. Benchekroun, A. Gillet and C. André, *Eur. J. Clin. Pharmacol.* 37, 85–90 (1989).
[12] J.M. Duthel, C. Mignot and J.J. Vallon, *Anal. Letts* 22, 2627–2636 (1989).
[13] J.M. Duthel, C. Mignot and J.J. Vallon, *Anal. Letts* 23, 799–817 (1990).
[14] K.Y. Tserng, *J. Pharm. Sci.* 72, 526–529 (1983).
[15] S. Floberg, B. Lindstrom and G. Lonnerholm, *J. Chromatogr.* 221, 166–169 (1981).
[16] B.D. Quimby and J.J. Sullivan, *Anal. Chem.* 62, 1027–1034 (1990).
[17] J.J. Sullivan and B.D. Quimby, *Anal. Chem.* 62, 1034–1043 (1990).
[18] D. Deruaz and J.M. Mermet, *Analusis* 14, 107–118 (1986).
[19] P.L. Wylie and R. Oguchi, *J. Chromatogr.* 517, 131–142 (1990).
[20] R.H. Greeley, *J. Chromatogr.* 88, 229–233 (1974).