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Gas chromatography–atomic emission detection for the measurement of isotopes Application to bioequivalence studies

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Abstract

This study was designed to demonstrate the ability of gas chromatography–atomic emission detection (GC–AED) to quantitatively measure amounts of labeled and unlabeled molecules when they are mixed together with both variable overall concentrations (labeled+unlabeled) and variable isotope ratios. To perform this study, simulations of bioequivalence trials were carried out using ^{13}C stable isotopically labeled molecules (SIL) coadministered with the unlabeled drug to act as biological internal standards. Various methodological approaches are shown and different methods of calculation developed for the quantitative determination of both SIL and unlabeled molecules. The pharmacokinetic parameters experimentally obtained are quite in accordance with the target values and GC–AED appears to be a valuable alternative to mass spectrometry for this kind of trial with concomitant use of labeled and unlabeled molecules.

Keywords: Isotopes; Caffeine

1. Introduction

The assessment of bioavailability and bioequivalence is of importance in drug development. Besides classically designed studies, the use of stable isotopically labeled molecules (SIL) has been shown to afford some advantages in bioequivalence trials. In order to avoid a biological shift of the subjects between the two sequences of such a study, the bioavailability of a given formulation may be compared with that of an intravenous dose of the SIL molecule administered simultaneously. More interesting is the use of a SIL molecule as a biological internal standard when the intra-individual variability in pharmacokinetic parameters is high and when the biological shift between the two phases of the bioequivalence trial is quantitatively important. According to this methodology it is possible to correct the pharmacokinetic parameters and especially the area under the plasma concentration curve (AUC) from the biological shift by simultaneous coadministration of a dose of the SIL drug (as an oral solution) with each of the formulations can be compared. The biological shift occurring in a subject will affect both the SIL drug and the unlabeled one, i.e. both the reference and the formulation to be tested. Comparison of the two formulations can then be carried out based on the ratio: AUC of the formulation to be

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tested/AUC of the SIL dose. The bioavailability factor (F) can be expressed as $F=f\times f'$, where f is the fraction of the analytical dose liberated from the pharmaceutical preparation and f' is the fraction of the dose escaping presystemic elimination. It is obvious that, in order to obtain accurate bioequivalence parameters, f' has to be constant during the two sequences of the bioequivalence assay. The use of a SIL biological internal standard allows monitoring of this potential shift, correction of the AUC data and gives accurate results. Thus the SIL coadministration method offers the following advantages: (1) reduction of intra-individual variations, (2) improvement of patient comfort [1,2], (3) decrease of patient number [2–4], (4) decrease of costs [5]. In such a methodological design both labeled and unlabeled molecules have to be measured from the same biological sample. That can be done by using a chromatographic separation coupled with a mass spectrometric detection. An alternative is the use of gas chromatography coupled to atomic emission detection (GC–AED). Stable isotopes can be detected using GC–AED. The detection of hydrogen (^{1}H) and deuterium (^{2}H) was reported for the first time by McLean et al. [6]. Other applications using these isotopes have been described [7–9]. The emission lines associated with atomic emission of hydrogen and deuterium with oxygen as reactant gas were respectively 656.30 nm and 656.04 nm. Nitrogen isotopes (^{14}N and ^{15}N) can be detected using the CN molecular bands located respectively at 421.46 nm (^{14}N) and 420.12 nm (^{15}N) with methane as reactant gas under optimized conditions [10].

The atomic emission detector with its photodiode array, allows the detection of ^{13}C [11]. A procedure developed by Quimby and Sullivan [12] calculates real time contributions of ^{12}C and ^{13}C from the CO molecular bands located respectively at 342.574 nm (^{12}CO) and 341.712 nm (^{13}CO). For carbon isotope analysis, oxygen and hydrogen are used as reactant gases. Sensitivity and accuracy of this type of ^{13}C detection have been widely reviewed [13,14]. The use of this ^{13}C detection followed by subtraction of the contribution of the natural ^{13}C abundance allows the ^{13}C metabolic pattern after administration of a ^{13}C SIL precursor to be obtained, as shown by Boukraa et al. [15].

The aim of this study was to demonstrate that GC–AED is an analytical method enabling accurate measurement of mixtures of labeled and unlabeled molecules where both the overall drug concentration and isotope ratio (SIL drug/unlabeled drug) vary. Thus, we used GC–AED to measure isotope ratios from samples obtained in a simulation of coadministration of a SIL molecule and its unlabeled counterpart. The SIL molecule was simulated to be administered by an extravascular route whereas the unlabeled drug was administered either by oral or by intravenous route.

2. Experimental

2.1. Chemicals

Caffeine and 3-isobutyl-1,[7^{13}C]methylxanthine were from Sigma-Aldrich (Saint Quentin Falavier, France), 1,3,7-[$^{13}CH_3$]caffeine from Tracer technologies (Somerville, USA), toluene from Merck (Darmstadt, Germany) and ethyl acetate from Carlo Erba (Milan, Italy).

2.2. Apparatus

The chromatographic separation was performed on an HP1 capillary column (25 m×0.32 mm I.D., film thickness=0.17 μm). Helium pressure was set at 300 kPa at the column head and the flow-rate kept constant during elution (1.08 ml min^{-1}). The injector temperature was 260°C and injections (1 ml) were made in the splitless mode (valve time=40 s). The oven temperature was programmed as follows: 100°C for 0.8 min, 12°C/min up to 230°C and 20°C/min up to 260°C for 2 min. The transfer line was set at 260°C.

The analytical system comprised an atomic emission detector equipped with a diode array spectrometer (HP 5921A) and a microwave induced plasma coupled to a chromatograph (HP 5890, series II plus). An automatic sampler (HP 7673A) and a workstation (HP 5895) completed the system. The mobility of the diode array allows to collect all the emitted wavelengths within the interval 160–800 nm. When coupled to a gas chromatograph, this detector is close to the ideal for the selective detection of elements [14] since the user can obtain

Table 1
Pharmacokinetic parameters used for the i.v.–oral simulation

	Oral route	i.v. route
Initial dose (DO) (mg)	200	200
Absorption rate (K_a) (h^{-1})	0.9	
Elimination rate (K_e) (h^{-1})	0.125	0.125
Volume of distribution (V_d) (l)	32.5	32.5
Systemic availability (F)	0.7	1
Total clearance (C_{lt}) ($l\ h^{-1}$)	4.06	4.06
AUC (mg $h\ l^{-1}$)	34.46	49.23
Maximum plasma level (C_{max}) (mg l^{-1})	3.13	
Time for plasma peak (t_{max}) (h)	2.55	

the whole emission spectrum recorded during the elution of each compound and can ascertain the presence of one particular element or isotope and thus avoid artifacts generated by the evolution of the continuous background. The monitored emission lines were: 348.424 nm for nitrogen, 342.574 nm for ^{12}C and 341.712 nm for ^{13}C.

The emitting source was a 99.9999% pure helium plasma generated inside a silica discharge tube (I.D.=1 mm). This tube was cooled by water and placed inside a Beenakker resonant cavity (microwave induced plasma: 2.75 GHz, 70 W). The overall flow-rate inside the detector was 36 ml min^{-1}.

The system was optimized in order not to require any further tuning [8]. It was also possible to program the addition of reactant gases to the plasma (O_2, 99.998% and H_2, 99.998%).

3. Simulation of the i.v–oral coadministration

The molecule chosen for the simulation was caffeine and its tri[$^{13}CH_3$] counterpart. In order to obtain analytical samples for GC–AED analysis, the simulation of a coadministration of 200 mg of unlabeled caffeine by i.v. route and 200 mg of ^{13}C-labeled caffeine by oral route was performed using the pharmacokinetic parameters shown in Table 1. The simulation allowed ten samples to be chosen by calculation of target concentrations from the theoretical pharmacokinetic parameters. Samples containing these labeled and unlabeled concentrations were prepared for analysis. From the caffeine concentrations it was possible to calculate the respective ^{12}C and ^{13}C concentrations of each sample considering that 1.1% of the overall carbon is ^{13}C and that in the labeled molecule three of the light carbon atoms are ^{13}C.

Fig. 1 shows the variations of the ^{12}C and ^{13}C concentrations as well as the evolution of the $^{13}C/^{12}C$ ratio vs. time. It can be observed that this ratio tends toward a constant value (0.22) obtained as

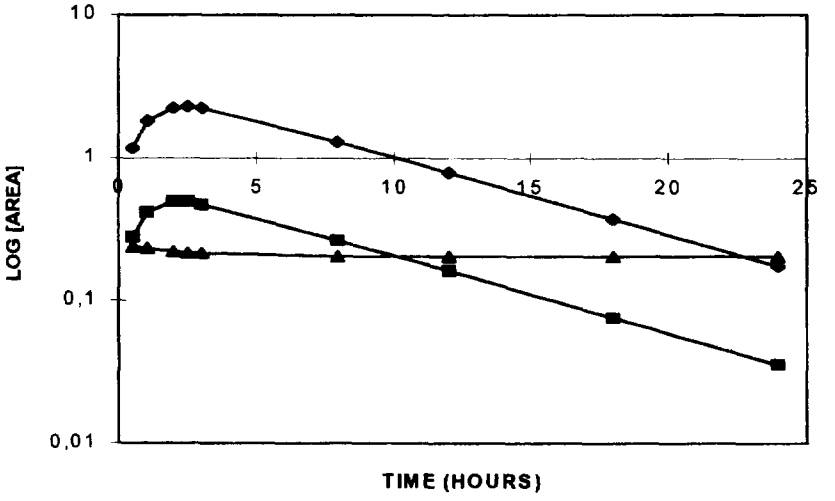

Fig. 1. ^{12}C (♦) and ^{13}C (■) concentrations after i.v.–oral coadministration and evolution of the $^{13}C:^{12}C$ ratio (▲) vs. time.

Table 2
Pharmacokinetic parameters used for the oral–oral simulation

	Oral route Labeled caffeine	Oral route Unlabeled caffeine
Initial dose (DO) mg	100	200
Absorption rate (K_a) (h^{-1})	1.20	0.90
Elimination rate (K_e) (h^{-1})	0.125	0.125
Volume of distribution (V_d) (l)	32.5	32.5
Systemic availability (F)	1	0.7
Total clearance (C_{tl}) ($l\ h^{-1}$)	4.06	4.06
AUC ($mg\ h\ l^{-1}$)	24.62	34.46
Maximum plasma level (C_{max}) ($mg \times l^{-1}$)	2.37	3.13
Time for plasma peak (t_{max}) (h)	2.10	2.55

soon as the absorption phase of the oral dose is completed.

4. Simulation of the oral–oral coadministration

In order to simulate a study of bioequivalence with an oral coadministration of a dose of the labeled molecule acting as a biological internal standard and allowing the correction of the intra-individual biological shift, the pharmacokinetic parameters shown Table 2 have been used.

As for the i.v.–oral coadministration, these parameters allowed simulation of the plasma concentration curves for both labeled and unlabeled caffeine after the simultaneous oral administration of a solution containing 100 mg of the labeled molecules ($F=1$) and 200 mg of unlabeled caffeine in a pharmaceutical formulation whose systemic bioavailability was $F=0.7$. Analytical samples containing both molecules were prepared from these simulations. Fig. 2 shows the variations of the ^{12}C and ^{13}C concentrations as well as the $^{13}C/^{12}C$ ratio vs. time. It can be observed that the ratio is rather constant and varies only between 0.24 and 0.20.

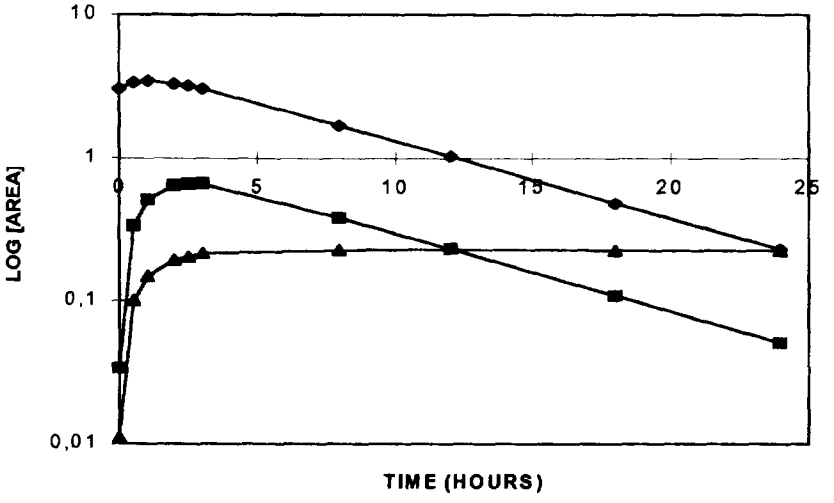

Fig. 2. ^{12}C (♦) and ^{13}C (■) concentrations after oral–oral coadministration and evolution of the $^{13}C:^{12}C$ ratio (▲) vs. time.

5. Methods of calculation

When a mixture of unlabeled and labeled molecules is analysed by GC–AED, the two isotopomers are coeluted from the chromatographic column. It is not possible to directly detect the proportion of analyte corresponding to the labeled molecule because of the natural abundance of the heavy isotope in the unlabeled molecule. The analytical response corresponding to the ^{13}C signal is the sum of the ^{13}C natural abundance of the unlabeled molecule and of the ^{13}C content of the labeled one. The same situation arises with ^{12}C or nitrogen. So we have developed two methods for the calculation of the isotope contents.

5.1. Method 1

M and M^* are the respective concentrations of unlabeled and labeled analytes and S_e the internal standard concentration (IBMX, isobutyl methyl xanthine). $S(^{12}C)$ and $S(^{13}C)$ are the areas under the analyte ^{12}C and ^{13}C chromatographic peaks respectively. $S_e(^{12}C)$ and $S_e(^{13}C)$ are the areas under the internal standard ^{12}C and ^{13}C chromatographic peaks. The measurement of the ^{12}C and ^{13}C signals from two calibration trials for M and M^* respectively allows the calculation of the parameters of four calibrations lines:

$$S(^{12}C)/S_e(^{12}C) = f([M]/[S_e]) = A1 \cdot [M]/[S_e] + B1$$

$$S(^{13}C)/S_e(^{13}C) = f([M]/[S_e]) = A3[M]/(S_e) + B3$$

$$S(^{12}C)/S_e(^{12}C) = f([M^*]/[S_e]) = A2 \cdot [M^*]/(S_e) + B2$$

$$S(^{13}C)/S_e(^{13}C) = f([M^*]/[S_e]) = A4 \cdot [M^*]/(S_e) + B4$$

When a mixture containing both M and M^* is analyzed, the overall signal for ^{12}C is:

$$S1 = (A1 \cdot [M]/[S_e] + B1) + (A2 \cdot [M]/[S_e] + B2) \quad (1)$$

and the overall 13C signal is:

$$S2 = (A3 \cdot [M^*]/[S_e] + B3) + (A4 \cdot [M^*]/[S_e] + B4) \quad (2)$$

The solutions for these equations for $[M]$ and $[M^*]$ are:

$$[M] = [S_e] \cdot (S1A4) - (S2A2)/(A1A4) - (A3A2) \quad (3)$$

$$[M^*] = (S_e) \cdot (S2A1) - (S1A3)/(A1A4) - (A3A2) \quad (4)$$

5.2. Method 2

If we consider $S(N)$ as the area under the chromatographic peak of the analyte (caffeine + labeled caffeine) measured from the nitrogen atomic emission line ($l = 348.424$ nm), and $S_e(N)$ the same signal from the internal standard, we can calculate two regression lines corresponding to:

$$S'1 = S(N)/S_e(N) = f(([M] + [M^*])/S_e) \quad (5)$$

$$S'2 = S(^{13}C)/S_e(^{12}C)/S_e(^{13}C)/S(^{12}C) = f([M^*]/([M^*] + (M))) \quad (6)$$

the corresponding equations are:

$$S'1 = A5 \cdot ([M] + [M^*])/S_e + B5 \quad (7)$$

$$S'2 = A6 \cdot ([M^*]/([M^*] + [M]) + B6 \quad (8)$$

From Eqs. (7,8), the unlabeled and labeled analyte concentrations can be calculated:

$$[M^*] = [S_e]'(S1 - B5) \cdot (S2 - B6)/(A5 \cdot A6) \quad (9)$$

$$[M] = [S_e] \cdot (S1 - B5) \cdot (A6 - S2 + B6)/(A5 \cdot A6) \quad (10)$$

6. Results and discussion

6.1. Coadministration i.v.–oral

6.1.1. Method 1

For the simulation, the following standard curves were calculated for caffeine concentrations ranging from 1.2 to 50 ng/ml.

$$S(^{12}C)/S_e(^{12}C) = f([M]/[S_e])$$

Table 3
Parameters of the regression lines $S(^{12}C)/S_e(^{12}C)=f([M]/[S_e])$ and $S(^{12}C)/S_e(^{12}C)=f([M^*]/[S_e])$

$S(^{12}C)/S_e(^{12}C)$	Unlabeled caffeine	Labeled caffeine
Slope	0.836 (A1)	0.687 (A2)
Intercept	0.083 (B1)	−0.028 (B2)
r^2	0.9986	0.9993
F	1.89	1.31

$$S(^{13}C)/S_e(^{13}C)=f([M]/[S_e])$$

$$S(^{12}C)/S_e(^{12}C)=f([M^*]/[S_e])$$

$$S(^{13}C)/S_e(^{13}C)=f([M^*]/[S_e])$$

Table 3 Table 4 show the parameters of the standard curves corresponding to unlabeled caffeine and caffeine labeled with three ^{13}C atoms on the methyl groups at positions 1, 3 and 7.

The test for linearity was performed by an analysis of variance on the regression and calculation of $F=MS_{lof}/MS_{pe}$ and comparison of the values obtained with tabulated values: F tab $_{(95\%,\ 3,\ 10)}=3.71$.

These results show that in the studied range of concentrations, the calibration curves for unlabeled and ^{13}C-labeled caffeine are quite linear. Plasma concentrations of both caffeine isotopomers were calculated using Eqs. (3,4). The correlations between measured concentrations and target values are excellent with slopes not significantly different from 1 and r^2 values of 0.9988 for both labeled (intravenous) and unlabeled caffeine (extravascular). Experimental values were processed to calculate the pharmacokinetic parameters using the SIPHAR software. Target values as well as model parameters are shown in Table 5.

These data fitted an open monocompartment model with first order elimination and absorption (extravascular).

Table 4
Parameters of the regression lines $S(^{13}C)/S_e(^{13}C)=f([M]/[S_e])$ and $S(^{12}C)/S_e(^{13}C)=f([M^*]/[S_e])$

$S(^{12}C)/S_e(^{12}C)$	Unlabeled caffeine	Labeled caffeine
Slope	0.451 (A3)	1.909 (A4)
Intercept	0.085 (B3)	−0.034 (B4)
r^2	0.9972	0.9977
F	2.95	3.37

6.1.2. Method 2

In the simulation using the measurement of nitrogen, ^{13}C and ^{12}C, the standard curves corresponding to Eq. (7) and Eq. (8) were calculated from standard mixtures of unlabeled and [$^{13}C_3$]-caffeine with a concentration ratio ranging from 0 to 85% of the ^{13}C-isotopomer. As previously, the test of linearity was performed by an analysis of variance on the regression and calculation of F. Table 6 gathers the parameters of these regression lines.

As for method 1, the correlation between measured and target values was excellent with slopes not significantly different from 1 and $r^2=0.9991$ for

Table 5
Target and model parameters calculated from labeled and unlabeled caffeine concentrations

	Target parameters	Model parameters	Difference(%)
Intravenous administration			
K_e (h^{-1})	0.125	0.129	3.2
V_d/F (l)	32.50	31.78	−2.2
AUC ($mg\cdot h\cdot l^{-1}$)	49.23	48.63	−1.2
Extravascular administration			
K_e (h^{-1})	0.125	0.128	2.4
K_a (h^{-1})	0.90	0.89	−1.1
V_d/F (l)	46.43	42.99	−7.4
AUC ($mg\cdot h\cdot l^{-1}$)	34.46	36.22	5.1
C_{max} ($mg\cdot l^{-1}$)	3.13	3.36	7.3
T_{max} (h)	2.55	2.53	−0.8
F (bioavailability)	0.70	0.74	5.7

Table 6
Regression parameters of method 2

$S(N)/S_e(N)$	
Slope	1.135 (A5)
Intercept	−0.105 (B5)
r^2	0.9982
F	2.54
$S(^{12}C)/S_e(^{12}C)/S_e(^{13}C)/S(^{13}C)$	
Slope	0.022 (A6)
Intercept	0.449 (B6)
r^2	0.9985
F	2.64

unlabeled caffeine (intravenous) and $r^2=0.9985$ for its ^{13}C isotopomer (extravascular). Experimental values fitted with an open monocompartment model whose parameters are gathered in Table 7.

6.2. Coadministration oral–oral

The analytical samples corresponding to the simulation of the oral–oral coadministration were processed according to method 1 after the measurement of the ^{12}C and ^{13}C responses. The correlation between measured concentrations and target values are excellent with slopes not significantly different from 1 and r^2 value of 0.9977 for unlabeled caffeine and of 0.9953 for its labeled counterpart. The pharmacokinetic parameters derived from these concentrations are gathered in Table 8.

These results are in good agreement with the target values calculated with the theoretical parameters.

7. Conclusion

The results of this study demonstrate that GC–AED is able to specifically detect and measure ^{13}C-labeled molecules simultaneously with their unlabeled counterparts. The simulations performed here show that these quantitative determination are possible either when the (labeled:unlabeled) ratio varies

Table 7
Target and model parameters calculated from labeled and unlabeled caffeine concentrations

	Target parameters	Model parameters	Difference(%)
Intravenous administration			
K_e (h^{-1})	0.125	0.128	2.4
V_d/F (l)	32.50	32.70	0.6
AUC ($mg \cdot h \cdot l^{-1}$)	49.23	49.81	1.2
Extravascular Administration			
K_e (h^{-1})	0.125	0.125	0
K_a (h^{-1})	0.90	0.93	3.3
V_d/F (l)	46.43	42.74	−7.9
AUC ($mg \cdot h \cdot l^{-1}$)	34.46	37.28	8.2
C_{max} ($mg \cdot l^{-1}$)	3.13	3.43	9.6
T_{max} (h)	2.55	2.47	−3.1
F (bioavailability)	0.70	0.74	5.7

Table 8
Pharmacokinetic parameters obtained after oral–oral coadministration (target values)

Extravascular administration	Labeled caffeine	Unlabeled caffeine
K_e (h^{-1})	0.131(0.125)	0.128(0.125)
K_a (h^{-1})	0.97(1.20)	0.87(0.90)
V_d/F (l)	58.41(65.00)	43.03(46.43)
AUC ($mg \cdot h \cdot l^{-1}$)	23.16(24.60)	34.64(34.46)
C_{max} ($mg \cdot l^{-1}$)	2.66(2.37)	3.31(3.13)
T_{max} (h)	2.47(2.10)	2.54(2.55)
F (bioavailability)	1 (ref.)	0.74(0.70)

(coadministration i.v.–oral) or when it remains rather constant (coadministration oral–oral) as well as when the overall amount of molecules detected (labeled + unlabeled) also varies. In this example of bioequivalence assessment, the results show that GC–AED allows the accurate determination of pharmacokinetic parameters when using a study design of coadministration of a SIL biological internal standard. The observed results are quite pertinent and coherent with target values. So, from the analytical point of view, GC–AED may be a valuable alternative to mass spectrometry for such studies or studies designed with the use of tracer doses of ^{13}C SIL molecules simultaneously with their unlabeled equivalent.

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