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A new application of bioorganometallics: the first simultaneous triple assay by the carbonylmetalloimmunoassay (CMIA) method

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Abstract

The first example of simultaneous triple carbonylmetalloimmunoassay (CMIA), a non-isotopic immunological method, is presented herein. This method, originally developed for mono-assays, uses various transition metal carbonyl complexes as labels and sensitive quantitation of these tracers by Fourier transform infrared (FT-IR) spectroscopy, thanks to the characteristic ν_{CO} bands appearing in the 1800–2200 cm^{-1} region. We show here that this method offers an advance in the challenging field of simultaneous multi-immunoassay, with the example of the assay of three antiepileptic drugs (carbamazepine, phenobarbital and diphenylhydantoin).

Keywords: Metal carbonyl tracer; Multi-immunoassay; Fourier transform IR spectroscopy; Therapeutic drug monitoring

1. Introduction

The CO ligand has played a unique role in the development of transition organometallic chemistry over the last century. In the early years, Mond's discovery [1] was at the origin of the first industrial application of a metal carbonyl complex, i.e. $Ni(CO)_4$, in the preparation of ultrapure nickel. Then, the work of Roelen [2] led to the first industrial application of metal carbonyls in homogeneous catalysis (olefin hydroformylation in the presence of $Co_2(CO)_8$). Later on, Fischer, while studying the reactivity of these complexes (such as $Cr(CO)_6$), discovered new functional groups such as metallocarbenes [3].

Since the pioneering work of Cotton [4], it has been well known that metal carbonyls have very particular features in the mid-IR spectral range. They display very strong bands in the 1800–2200 cm^{-1} region, where few other vibrators absorb. All the $M(CO)_x$ entities give a set of characteristic and intense ν_{CO} bands, the number and the position of which depend on their nature, local symmetry and electronic environment.

The implementation of multi-immunoassays for the simultaneous assay of several analytes is recognized as one of today's bioanalytical challenges [5]. In this manner, assay procedures would be simplified and require smaller blood samples, providing a saving of both time and money and improved service to the patient. Although time-resolved fluoroimmunoassay has raised hopes with an instance of the simultaneous multi-assay of four substances [6], only double assays are now routinely developed [7]. In fact, the stumbling block to the implementation of multi-immunoassays lies in the difficulty in finding combinations of labels that emit distinct and simultaneously analyzable signals of close intensities.

We suggest that the use of metal carbonyl complexes as labels could bring a suitable response to this issue. The non-isotopic competitive immunoassay method named carbonylmetalloimmunoassay (CMIA) exploits the particular features of metal carbonyl complexes in the mid-IR spectral range. This method has been successively applied to the steroid hormone cortisol [8],

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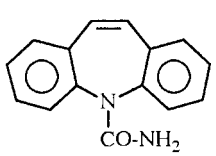
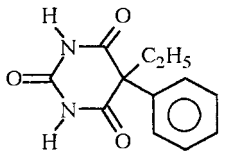
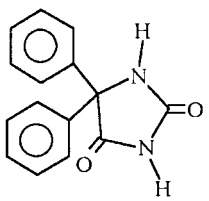


Carbamazepine



Phenobarbital



Diphenylhydantoin

Fig. 1. The three antiepileptic drugs.

and to several antiepileptic drugs [9]. Dual CMIAs of antiepileptic drugs were also successfully implemented [10]. We report here that this method can be extended to the simultaneous immunoassay of three drugs, carbamazepine (CBZ), phenobarbital (PB) and diphenylhydantoin (DPH) shown in Fig. 1 and often prescribed as a cocktail in the treatment of epileptic seizures.

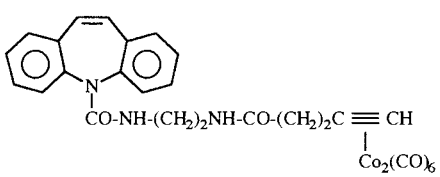
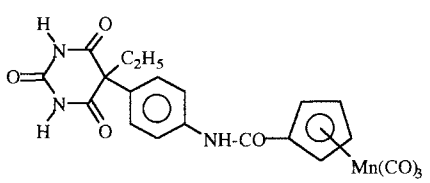
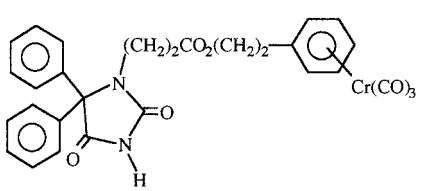
2. Results and discussion

To develop an immunoassay, one must first obtain a tracer that is a derivative of the analyte containing the label and second, an antibody that is able to specifically recognize both the analyte (the substance to be quantified) and the tracer. For a multi-immunoassay, it is also essential that the protagonists of the assay, i.e. the tracers, the drugs to be assayed and their specific antibodies, do not exhibit any cross-reaction.

In the present case, for low-molecular-weight compounds (i.e. haptens) devoid of antigenic properties, production of polyclonal antibodies against CBZ [9c], PB [11] and DPH [9b] was achieved by injection into rabbits of suitable immunogens obtained by coupling of drug derivatives to bovine serum albumin, a high-molecular-weight molecule. These antibodies used in the mono-CMIA of each of these drugs showed no cross-reactivity with the other two drugs, nor with the other two tracers.

Metal carbonyl tracers **A**, **B** and **C**, respectively derived from CBZ [12], PB [13] and DPH [9b] were obtained by conjugation of drug derivatives with suitably functionalized metal carbonyl complexes. Their

Table 1
Infrared characteristics of the metal carbonyl complexes in the ν(CO) region [a]

Metal carbonyl complex		ν(CO) (cm^{-1})	Molar extinction coefficient	Minimum quantity detectable (pmol) [b]
	**A**	2095, 2058 [c], 2032	3355 [d]	0.2
	**B**	2033 [c], 1958	1212	0.6
	**C**	1973 [c], 1899	1542 [d]	2

[a] IR spectra (20 scans, 4 cm^{-1} resolution) recorded in $CHCl_3$ on an MB 100 FT spectrometer equipped with an InSb detector and a light-pipe cell, 20 mm pathlength, filling volume 30 μl [20].

[b] Quantity of tracer having an absorbance equal to twice that of the noise level.

[c] Analytical band of the complex (band used to performed quantitative analysis).

[d] Measured in CCl_4.

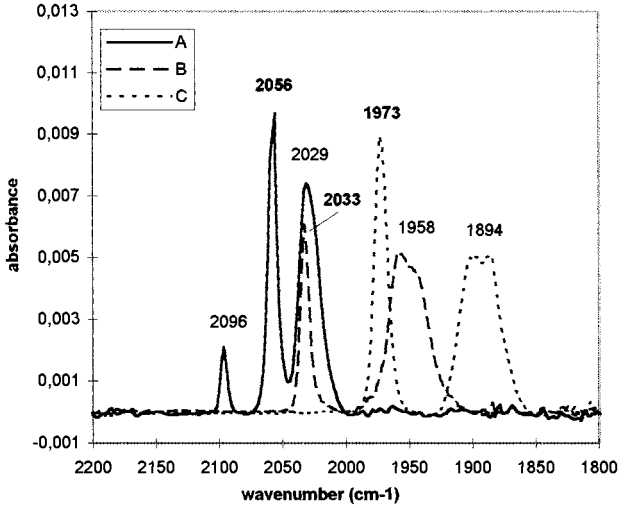


Fig. 2. Superimposition of the IR spectra in $CHCl_3$ of **A** (30 pmol), **B** (60 pmol) and **C** (60 pmol) in the ν_{CO} region.

structure is depicted in Table 1. They include different metal carbonyl fragments, an alkyne–$Co_2(CO)_6$ entity for CBZ, a cymantrenyl entity for PB and a benchrotrenyl entity for DPH. When the IR spectra of the three tracers in the ν_{CO} region (in $CHCl_3$) are superimposed, it can be seen that they differ in the number, the position and the relative intensities of the bands (Fig. 2). Complex **A** displays five bands (three of which are totally overlapped) in agreement with the local C_{2v} symmetry of the metal carbonyl group [14], while compounds **B** and **C** displays two bands, confirming to the local C_{3v} symmetry of the metal tricarbonyl moiety [15].

We have previously shown that each ν_{CO} band of the three complexes obeyed Beer's law in the picomole range [10]. However, for each complex, we identified one analytical band, providing the best precision and the highest sensitivity of analysis [16]. For **A**, it is the B_1 mode at 2058 cm^{-1}, for **B** and **C**, the A_1 mode at 2033 and 1973 cm^{-1}, respectively. Quantitation of each individual metal carbonyl tracer was achieved by direct measurement of the absorbance of their analytical band. This univariate method provided the following calibration equations for complexes **A**, **B** and **C** in the 10–100 pmol range:

$$Q(\mathrm{A}) = 331 \times A^{\mathrm{A}}_{2058} + 1.66 \quad (1)$$

$$Q(\mathrm{B}) = 10\,672 \times A^{\mathrm{B}}_{2033} - 1.41 \quad (2)$$

$$Q(\mathrm{C}) = 6711 \times A^{\mathrm{C}}_{1973} - 5.13 \quad (3)$$

where $Q(\mathrm{X})$ is the quantity of tracer X in pmol and A^{X}_{Y} is the absorbance of X at the selected wavelength Y.

The limit of detection of each tracer was found to be dependent on the value of the extinction coefficient ε of the analytical band and also on the position of this band. The lowest detection limit was reached with complex **A** (Table 1), whereas the highest value was observed for **C**, although its extinction coefficient is slightly higher than that of **B**. Actually, the analytical band of **C** is located in a spectral region where the noise is higher owing to interference caused by several water vapor bands that appear below 2020 cm^{-1}.

In order to perform a simultaneous multi-immunoassay, it is necessary to address the problem of quantitation of several metal–carbonyl tracers in mixtures. The selection of compatible metal–carbonyl entities becomes essential. One can choose entities comprising different metals as we have done with the mixture **A** + **B** + **C**. Alternatively, one can induce shifts of the ν_{CO} bands by adding substituents to the Cp ring and/or changing ligands in the cymantrenyl graft [17]. Another approach was to use the drug diphenyl hydantoin as a ligand of the $CpFe(CO)_2$ graft [18].

The ideal case is met when no overlap of the analytical bands is observed, as for the mixture **A** + **C**. However, a partial overlap is most frequently observed. In this latter case, measurement of absorbances at two different wavenumbers, more precisely at the position of the analytical band of the compounds allowed quantitation of both substances [10].

Mixtures of the three metal–carbonyl complexes **A**, **B** and **C** display more severe band overlaps (see Fig. 2). Although the analytical band of **A** (at 2058 cm^{-1}) is free, the analytical band of **B** (2033 cm^{-1}) is completely buried under the 2032 cm^{-1} band of **A** and the analytical band of **C** (1973 cm^{-1}) partially overlaps the 1958 cm^{-1} band of **B**. Nevertheless, measurement of absorbances at 2058, 2033 and 1973 cm^{-1} allowed prediction of the quantities of the three tracers in mixtures by applying a univariate method as follows. We first established the following equations linking the absorbance A^{X}_{Y} of complex X alone at the wavelength Y of its analytical band to the absorbance of the mixtures A^{M}_{Y}:

$$A^{\mathrm{A}}_{2058} = A^{\mathrm{M}}_{2058} \quad (4)$$

$$A^{\mathrm{B}}_{2033} = A^{\mathrm{M}}_{2033} - 0.76 \times A^{\mathrm{M}}_{2058} \quad (5)$$

$$A^{\mathrm{C}}_{1973} = A^{\mathrm{M}}_{1973} - 0.06 \times A^{\mathrm{M}}_{2033} + 0.046 \times A^{\mathrm{M}}_{2058} \quad (6)$$

Thus, combination of Eqs. (1) and (4), Eqs. (2) and (5) and Eqs. (3) and (6) led to the following equations linking the quantity of each complex $Q(\mathrm{X})$ to the absorbance of the mixtures A^{M}_{Y}.

$$Q(\mathrm{A}) = 3311 \times A^{\mathrm{M}}_{2058} + 1.66 \quad (7)$$

$$Q(\mathrm{B}) = 10\,672 \times A^{\mathrm{M}}_{2033} - 8111 \times A^{\mathrm{M}}_{2058} - 1.41 \quad (8)$$

$$Q(\mathrm{C}) = 6711 \times A^{\mathrm{M}}_{1973} - 403 \times A^{\mathrm{M}}_{2033} + 309 \times A^{\mathrm{M}}_{2058} + 5.13 \quad (9)$$

The precision of prediction, expressed as SEP (standard error of prediction, see formula in the Section 3), of quantities of complexes **A**, **B** and **C** in mixtures in the 20–100 pmol range was of 3.2, 5.7 and 4.0 pmol,

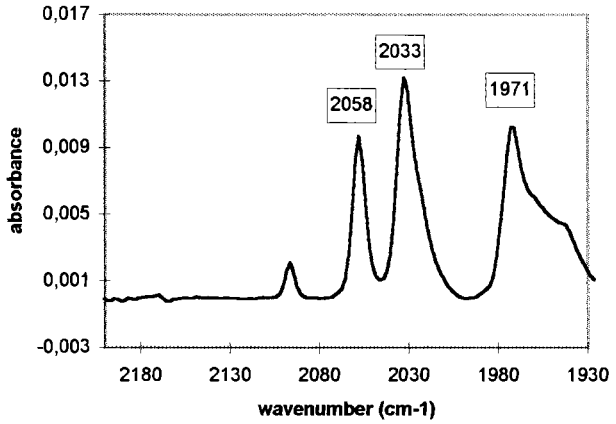


Fig. 3. IR spectrum in $CHCl_3$ of a mixture of **A** (30 pmol), **B** (60 pmol) and **C** (60 pmol) in the ν_{CO} region.

respectively (number of samples: nine). Alternatively, multivariate methods based on classical least-squares or partial least-squares algorithms were successfully applied [19].

Once the reactants necessary to the assay and the quantitative analysis method were both in place, we attempted the triple CMIA of the antiepileptic drugs. The procedure was to mix fixed amounts of the three antibodies, the three tracers **A**, **B** and **C** and increasing amounts of the three analytes, in a manner similar to that used in the single immunoassay. After 2 h, separation of the free fraction of tracers from the fraction of tracers bound to the antibodies was achieved by extraction with an organic, non-miscible solvent (diisopropylether). After evaporation of the organic solvent under vacuum, the samples were analyzed by FT-IR spectroscopy in a specially designed light-pipe cell [20]. In the ν_{CO} region, the resulting spectra were the sum of the spectra of the three metal carbonyl complexes (Fig. 3). Quantitation of the three tracers in each sample was done by the univariate method described above. From

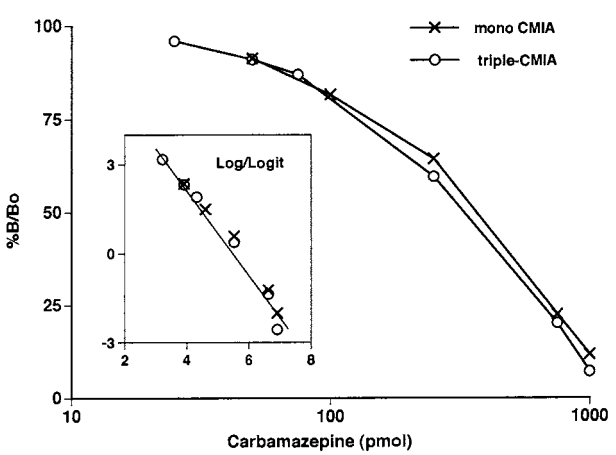


Fig. 4. Superimposition of the standard curves of CBZ in mono and triple CMIA. Inset: linearization of the curves.

these data, three standard curves (plot of bound fraction % B/B_0 vs. quantity of analyte) were generated.

Validation of multi-CMIA was performed by comparing the standard curves generated for each drug by mono and triple CMIA. The superimposition of the standard curves of CBZ obtained in the single and triple assay formats is shown in Fig. 4. As one can see, they are very close. Identical results were obtained for the two other drugs.

The log–logit transformation [21] was applied to the standard curves of each tracer obtained by the single and triple assays. This transformation allowed a good linear fit of the data in all cases as observed by the equations reported in Table 2. Moreover, the comparison of these equations indicates a very similar response of the three analytes in the single and triple assay formats, thus demonstrating the feasibility of the approach.

We have thus demonstrated the viability of an assay capable of simultaneously predicting the concentration of three drugs from a single sample, in one step and requiring no technical modification of the equipment used in single assays. The CMIA has thus broken the two-analyte boundary of simultaneous multi-immunoassays, this being the upper limit for routine tests. The use of more sophisticated mathematical methods should allow us to analyze mixtures of more than three analytes. These results have opened a new and highly promising area of application for metal carbonyl complexes.

3. Experimental

3.1. FT-IR analysis of mixtures of *A*, *B* and *C*

Nine mixtures of known quantities $Q(X)$ of tracers **A**, **B** and **C** ranging from 20 to 100 pmol were prepared in ethanolic solutions and put into 1.5 ml microtubes (Eppendorf). Solvent was evaporated under vacuum in a Speed Vac concentrator (Savant). IR samples were obtained by dissolving the residues in 30 μl of spectroscopic grade $CHCl_3$. IR analysis was performed on an MB 100 FT-spectrometer (Bomem) equipped with an InSb detector and a microbeam setup. Twenty scans were coadded at a resolution of 4 cm^{-1}. The sampling cell consisted of a 20 mm path length gold light-pipe with an internal volume of 23 μl [20]. The absorbances at 2058, 2033 and 1973 cm^{-1} were serially measured using the 'trend line analysis' function included in the Bomem Easy software. The predicted quantities of each tracer Q_P^X were calculated by applying Eqs. (7)–(9). The standard error of prediction, SEP, was determined as follows:

$$SEP = (1/9\Sigma(Q_P^X - Q^X)^2)^{1/2} \text{ in pmol.}$$

Table 2
Linearization of the standard curves by the log–logit transformation [a]

Analyte	CBZ	PB	DPH
Single assay	$Y = -1.42X + 8.07$ ($r^2 = 0.981$)	$Y = -0.79X + 3.64$ ($r^2 = 0.972$)	$Y = -1.04X + 6.10$ ($r^2 = 0.981$)
Triple assay	$Y = -1.49X + 8.21$ ($r^2 = 0.985$)	$Y = -0.67X + 3.34$ ($r^2 = 0.960$)	$Y = -1.00X + 5.99$ ($r^2 = 0.970$)

[a] $X = \ln(Q)$, $Y = \ln\left(\frac{\% B/B_0}{100 - \% B/B_0}\right)$.

3.2. Chemicals

Carbamazepine was a gift from Ciba–Geigy, the sodium salt of phenobarbital was obtained from Cooper de Melun (Melun, France) and diphenylhydantoin was purchased from Sigma.

3.3. Metal carbonyl tracers

The cobalt carbonyl complex of carbamazepine (**A**), the manganese-labeled phenobarbital (**B**) and the diphenylhydantoin chromium tricarbonyl complex (**C**) were synthesized as previously described [9b,12,13]. Stock solutions of these complexes (1×10^{-3} M for **A** and **C**, 4.3×10^{-4} M for **B**) were prepared in absolute ethanol. These solutions are stable for several months provided they are stored in the dark at about -20°C. Successive dilutions were performed in ethanol just prior to use.

3.4. Buffer

The phosphate buffer (pH 8.8) used for the CMIA analysis was as follows: Na_2HPO_4, 12 H_2O 30 mM, NaCl 160 mM, sodium azide 30 mM, glycerol 10% v/v.

3.5. Specific antibodies

The polyclonal *anti*-carbamazepine, *anti*-phenobarbital and *anti*-diphenylhydantoin antisera were produced in 'Fauves de Bourgogne' rabbits as previously described [9b–c,11]. Serum aliquots (150 μl fractions) were kept at -20°C prior to use.

3.6. Mono-immunoassay by CMIA

3.6.1. Mono-immunoassay of carbamazepine

Fractions of 500 μl of CMIA buffer (adjusted to a final percentage of 28% in ethanol) containing 50 μl of a dilution of *anti*-carbamazepine antibody equal to the titer value (defined as the dilution of antibody binding 50% of the tracer), 30 pmol in 20 μl of the organometallic tracer **A** and 5–50 μl of various quantities of carbamazepine (25–2000 pmol) were incubated at room temperature for 1 h. Separation of the free and bound fractions was performed by solvent extraction with 1 ml of diisopropyl ether saturated by buffer. 750 μl samples of the organic phase that contained the free tracer were withdrawn and transferred immediately to 1.5 ml microtubes. The solutions were quickly evaporated to dryness on a Speed Vac concentrator. Samples for IR analysis were obtained by dissolving the dry residues in 30 μl of $CHCl_3$ and the IR spectra were immediately recorded and processed as indicated above. Quantification of free amount of **A** (F) was performed by applying Eq. (1). The total quantity of tracer (T) was the quantity found following extraction of a 500 μl fraction containing an identical quantity of tracer but no antibodies. The bound fraction (B) was calculated from the difference ($T - F$). B_0 was designated as the B value obtained in the absence of carbamazepine. Competition curves were then obtained by plotting the $\% B/B_0$ ratios against the amount of carbamazepine added.

3.6.2. Mono-immunoassay of phenobarbital

The protocol used was the same as for carbamazepine except that incubation was performed in the presence of *anti*-phenobarbital antibodies, 60 pmol of the metal carbonyl tracer **B** (in 20 μl of ethanolic solution) and 5–50 μl of various quantities of phenobarbital (25–3000 pmol). Quantitative analysis was performed by applying Eq. (2).

3.6.3. Mono-immunoassay of diphenylhydantoin

The protocol used was the same as for carbamazepine except that incubation was performed in the presence of *anti*-diphenylhydantoin antibodies, 60 pmol of the metal carbonyl tracer **C** (in 20 μl of ethanolic solution) and 5–50 μl of various quantities of diphenylhydantoin (25–2000 pmol). Quantitative analysis was performed by applying Eq. (3).

3.7. Triple immunoassay by CMIA

Fractions of 500 μl of CMIA buffer (adjusted to a final percentage of 28% in ethanol) containing the three antibodies (3×50 μl; dilutions equal to the titer value), the three metal carbonyl tracers (30 pmol of **A**, 60 pmol of **B** and **C** in 3×20 μl of ethanolic solutions), and 5–50 μl of various quantities (25–3000 pmol) of the three compounds to be tested were incubated at room temperature for 1 h. Separation of the free and bound fractions was performed by solvent extraction with 1 ml

of diisopropyl ether saturated by buffer. 750 μl samples of the organic phase that contained the three free tracers were withdrawn and transferred immediately to 1.5 ml microtubes. The solutions were quickly evaporated to dryness on a Speed Vac concentrator. Samples for IR analysis were obtained by dissolving the dry residues in 30 μl of $CHCl_3$ and the IR spectra were immediately recorded and processed as indicated above. Quantitative analysis of the mixtures of the tracers was performed by using Eqs. (7)–(9).

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