ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
21 (1999) 625–633

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Carbonyl metallo immuno assay: a new application for Fourier transform infrared spectroscopy☆

Anne Vessières [a,*], Michèle Salmain [a], Pierre Brossier [b], Gérard Jaouen [a]

[a] *Ecole Nationale Supérieure de Chimie de Paris, Laboratoire de Chimie Organométallique-UMR CNRS 7576, 11 rue Pierre et Marie Curie, F-75231 Paris Cedex 05, France*

[b] *Université de Bourgogne, Facultés de Médecine et Pharmacie, 7, Boulevard Jeanne d'Arc, 21033 Dijon Cedex, France*

Received 15 May 1998; received in revised form 30 September 1998; accepted 24 October 1998

Abstract

We describe here the development of a new, non-isotopic immunological assay termed CMIA (carbonyl metallo immunoassay) that uses metal carbonyl complexes as tracers and Fourier transform infrared spectroscopy (FT-IR) as the detection method. This assay is based on the particular spectral features of these complexes, which show very strong absorption bands in the 1800–2200 cm^{-1} spectral range where proteins and organic molecules do not absorb. In Section 1, the optimisation of the quantitative detection of these tracers is detailed. In Section 2, the implementation of mono-CMIA is described, including the CMIA assays of three antiepileptic drugs (carbamazepine, phenobarbital, phenytoin). Finally, extension to the simultaneous double- and triple-CMIA of these drugs is reported.

Keywords: Non-isotopic immunoassay; Antiepileptic drugs; Fourier transform infrared spectroscopy; Metal carbonyl tracer; Multi-immunoassay

1. Introduction

Since the early 1960s, the use of immunological tests has revolutionised clinical chemistry and biochemistry. At that time, most of these assays were radioimmunoassays employing radioisotopes (^{125}I, ^{3}H) as labels. However, because of the hazards associated with the use of radioactivity, ever since the early 1970s, we have witnessed the development and commercialisation of a number of non-isotopic immunological methods for the assay of a wide variety of clinically meaningful analytes. The most widespread methods now use enzymes, fluorescent, chemi- and bio-luminescent probes as labels associated to analytical methods such as colorimetry, fluorescence spectroscopy or luminometry [1].

Although the non-isotopic methods have their specific advantages, they also show limitations. For example, fluorescence interference is often encountered in the UV-visible region due to natu-

☆ Presented at the Drug Analysis 1998 meeting, Brussels, Belgium, 11–15 May 1998.

* Corresponding author. Tel.: +33-144-276-729; fax: +33-143-260-061.

E-mail address: vessiere@ext.jussieu.fr (A. Vessières)

0731-7085/99/$ - see front matter
PII: S0731-7085(99)00169-7

rally occurring or other contaminating fluorophores [2]. Consequently, there is a continuous effort to develop new immunological methods. In our particular case, we have been exploring the potential in the biomedical field of transition metal carbonyl labels detected by Fourier transform infrared spectrometry [3]. Our work in this area resulted in the development of a new non-isotopic immunoassay called carbonyl metallo immuno assay (CMIA).

CMIA is an heterogeneous, competitive-type non-isotopic immunoassay, employing metal carbonyl complexes as labels. The free and bound fractions of the tracer are separated by solvent extraction, and the tracer is quantified using Fourier transform infrared spectroscopy. In this article, we describe the development of CMIA of haptens and its extension to the simultaneous multi-assay of two and three antiepileptic drugs, carbamazepine, phenobarbital and phenytoin (DPH).

2. Optimisation of the analysis of metal-carbonyl labels by FT-IR spectroscopy

The first question to address was whether FT-IR spectroscopy would be a sufficiently reliable and sensitive method for biomedical-grade quantitative analysis. This was not a foregone conclusion. In fact, infrared spectroscopy has only recently begun to be used as an analytical tool, a development linked to the arrival on the market of benchtop FT-IR spectrometers [4]. For this reason, examples of FT-IR as an analytical technique for medical applications are rare. Only few examples, all recent, such as glucose analysis in blood [5], and the composition of kidney stones [6], have been published. The reasons for the lack of development of infrared techniques in the biomedical area can be summed up by the following two points: first, biological media are usually aqueous media which absorb strongly in the mid-infrared spectral range, and second, the quantities to be analysed tend to be very small, in the order of picomoles, and absolutely out of range with the former dispersive IR spectrometers. Even with the recent FT-IR technology, this level of sensitivity is not achieved routinely on benchtop apparatus, although these machines do have the advantages of affordability and ease of use.

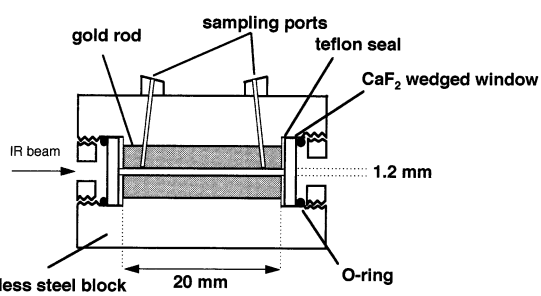

Fig. 1. Schematic drawing of the gold light-pipe cell.

Our first challenge was therefore to optimise the sensitivity of the FT-IR spectrometer to a level that would be compatible with the requirements of immunoanalysis of drugs [6].

The factors we were able to manipulate are detailed below. Some of them involved the hardware itself, beginning with selection of the detector. We found that it was essential to use a liquid nitrogen-cooled detector of semi-conducting material, such as an MCT (mercury–cadmium–telluride) or InSb (indium antimonide) detector. This provided a level of sensitivity that was 20 times greater than a thermal detector such as the DTGS (deuterium triglycine sulfate) detector that is the standard in most FT-IR spectrometers. An analysis solvent, such as carbon tetrachloride or chloroform, was selected to be transparent in the mid-infrared range. Finally, we took advantage of the fact that, in solution, the absorbance obeys Beer's law ($A = \varepsilon \times l \times c$) where ε is the extinction coefficient of the compound ($m \times l^{-1} \times \mathrm{cm}^{-1}$), l is the optical pathlength of the measurement cell (cm) and c is the concentration of compound in the sample ($m \times l^{-1}$). Thus, an increase in the sensitivity of the technique should be obtainable simply by increasing the optical pathlength. It was necessary in our case to take into account the specific constraints of clinical analysis, in which samples may consist of only a few microliters of dilute solution. Our solution was to have a light-pipe cell constructed (Fig. 1), consisting of a gold tube with a pathlength of 2 cm for a fill volume of only 30 μl [8]. This cell proved extremely well

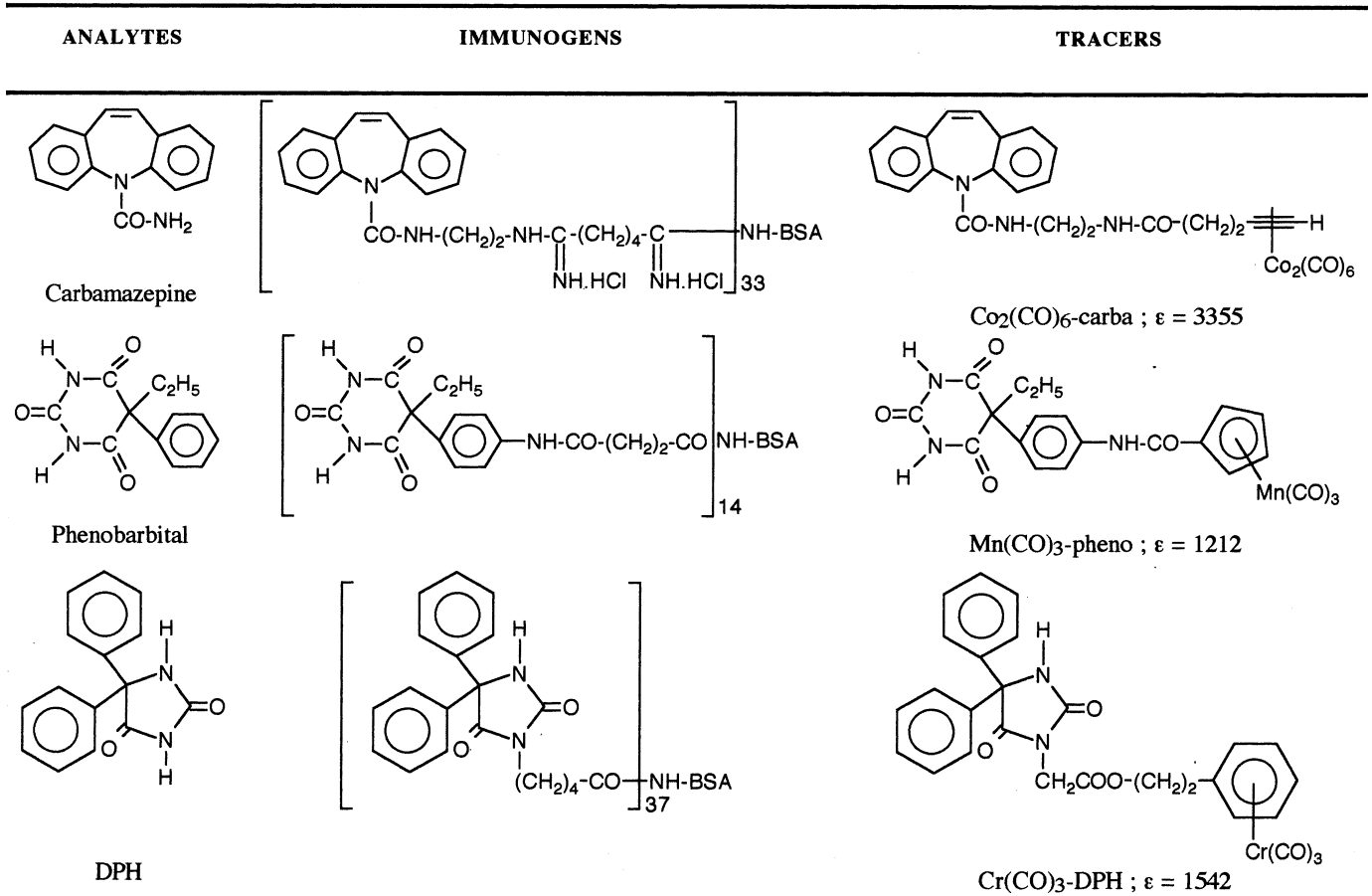

Fig. 2. Chemical formulae of the antiepileptic drugs, their immunogens and their metal carbonyl tracers. ε, extinction coefficient of the tracers ($1 \times m^{-1} \times cm^{-1}$).

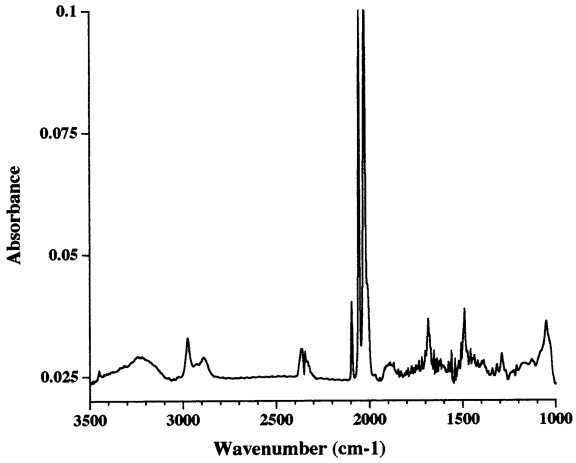

Fig. 3. IR spectra (20 scans, 4 cm^{-1} resolution) of $Co_2(CO)_6$-carba in CCl_4 recorded on a MB100 FT-spectrometer (Bomem) equipped with an MCT detector.

adapted for our application compared to standard infrared cells with an optical pathlength of 0.1 mm, we improved sensitivity by a factor of 200. Once the hardware was optimised, we turned our attention to the nature of the metal carbonyl entity. By definition, an organometallic complex is a molecule containing covalent metal–carbon bonds. Bonds between the metal, which is usually in a low oxidation state, and the carbon ligands involve π-electrons such as those found in triple bonds or aromatic rings which will interact with the empty orbitals of the metal atoms. These bonds are stabilised by back-donation from the filled metal d orbitals into the ligands π*-orbitals [9]. Metal-carbonyl complexes are a particular family of organometallic compounds with carbon monoxide (CO) ligands coordinating the transition metal.

In our case, we were interested in the assay of the three antiepileptic drugs depicted in Fig. 2. The three medications are carbamazepine, phenobarbital and DPH. These substances were chosen because they are assayed on a large scale in hospitals, owing to the fact that the margin between the toxic and effective doses is small, and the drugs may be metabolised differently from one individual to another [10]. These substances are often administered together. For each of these three drugs, we selected different metal carbonyl probes and prepared their corresponding organometallic complexes. For carbamazepine, we synthesised a dicobalt hexacarbonyl cluster of an acetylenic derivative [11]. For phenobarbital, a cyclopentadienyl $Mn(CO)_3$ entity derivative was selected [12], and for DPH we chose to attach an arene-$Cr(CO)_3$ group [13]. Although these organometallic entities do bring some steric crowding as compared to the free hapten to which they are attached, this crowding is less than for most fluorescent chelates, such as the europium chelates that are used as labels in many non-isotopic immunoassays.

We chose to work with metal–carbonyl complexes because they display very particular features in the mid-infrared spectral region. Fig. 3 shows the infrared spectrum of the carbamazepine tracer. As expected, the organic part of the compound exhibits peaks between 800 and 1800 cm^{-1} and ~ 3000 cm^{-1}, but most markedly, characteristic strong peaks assigned to the stretching vibration modes of carbonyl ligands ν_{CO} appear in the 1800–2200 cm^{-1} region. These peaks are eight to ten times more intense than all the other peaks in the spectrum and, most importantly, they occur in a region where most of the organic molecules do not absorb. This feature, which is due to the artificial nature of CO ligands, is shared by all metal carbonyl complexes.

These labels have another very important advantage over other families of labels, (Fig. 4). The superimposition of the infrared spectra of the three tracers show that they are quite different from one complex to another. Indeed, the position, intensity and number of ν_{CO} modes depend on the nature of the metal and on the local symmetry of the metal carbonyl entity. For the $Co_2(CO)_6$ cluster, one would expect five peaks, owing to the C_{2v} group symmetry of this entity [14], but in solution only three bands are seen, because the 2027 cm^{-1} peak is in fact the envelop of three vibration modes. For the $Mn(CO)_3$ and $Cr(CO)_3$ groups, each complex are expected to give two characteristic peaks owing to the C_{3v} symmetry, one sharp (of the symmetric A_1 mode) and one broader (of the anti-symmetric E mode) [15], and indeed two bands are detected. Furthermore, for a given metal carbonyl moiety, we

noticed that changing the bioligand has a minimal effect on the absorbance of the ν_{CO} modes [7].

As our goal was the quantitative analysis of these complexes, we looked for possible proportionality between the absorbance of the characteristic peaks in the 2000 cm^{-1} region, and the quantity of tracer present in the sample, as expected from Beer's law. When the absorbance of each of the peaks was plotted against the quantity of complex, straight lines were obtained, confirming that peak heights were in fact proportional to the quantity of tracer. However, the detection limit was different depending which peak was used. For example, the highest sensitivity was reached with the narrow intense peak at 2056 cm^{-1} for $Co_2(CO)_6$-carba. The detection limit was 300 fmol of tracer. This band was then taken as the analytical band for further calculations. Similarly, the bands at 2032 and 1971 cm^{-1} were taken as the analytical bands for tracers $Mn(CO)_3$-pheno and $Cr(CO)_3$-DPH, respectively. We noticed a variation in the minimum tracer quantities detectable. This value depends not only on the absorptivity of the tracer (cf ε values of the tracers in Fig. 2) but also on the compensation of the vibration–rotation bands of atmospheric water vapour that appear throughout the 2020–1800 cm^{-1} region. $Co_2(CO)_6$ tracer gave a slightly lower detection limit than $Mn(CO)_3$ and $Cr(CO)_3$ tracers [7]. However, for all the tracers studied, the sensitivity is well-adapted to drug immunoassay.

3. Implementation of mono-CMIA

At this stage, we had tracers for each of the drugs we wished to assay and an analytical technique to quantify them. Regarding the third partner of any immunoassay, i.e. the antibody, we chose to work with polyclonal antibodies. These specific antibodies were raised in rabbits by im-

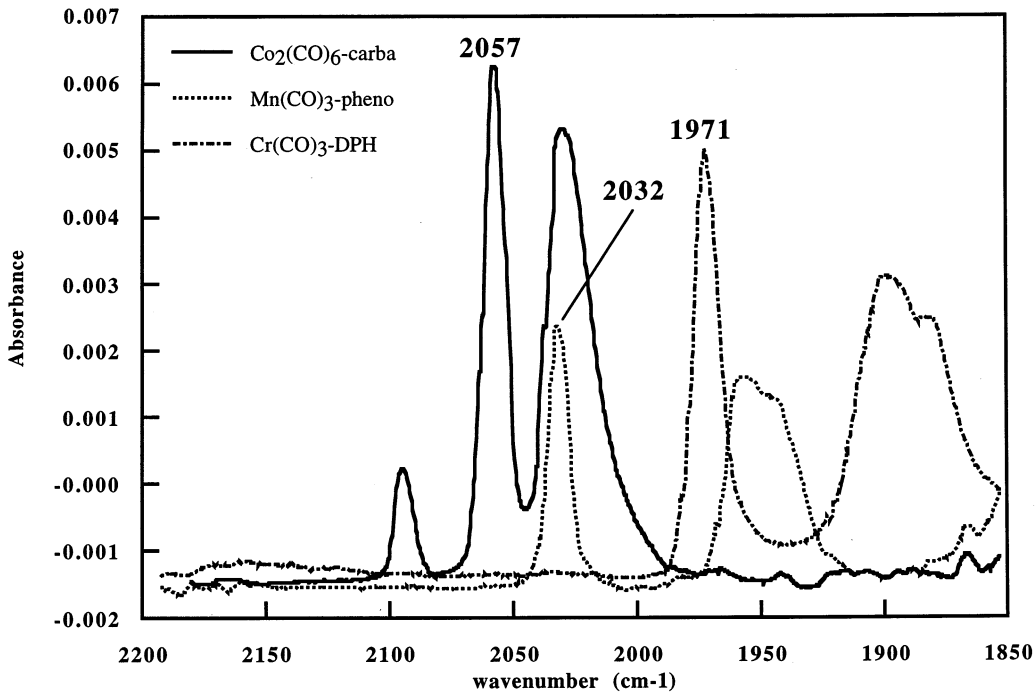

Fig. 4. Superimposition of the IR spectra (20 scans, 4 cm^{-1} resolution) of the three metal carbonyl tracers in solution in chloroform recorded on a MB100 FT-spectrometer (Bomem) equipped with an InSb detector. Light-pipe cell. Wavenumbers of the analytical bands are shown.

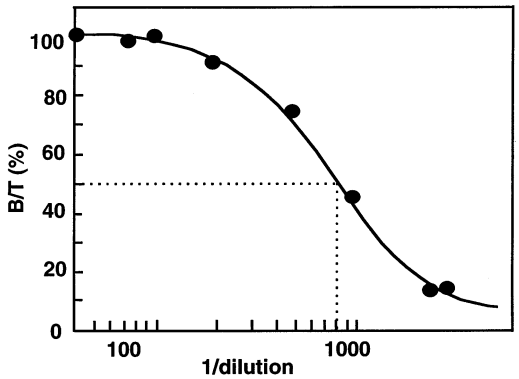

Fig. 5. Antiserum dilution curve by CMIA obtained in the presence of 30 pmol of $Co_2(CO)_6$-carba.

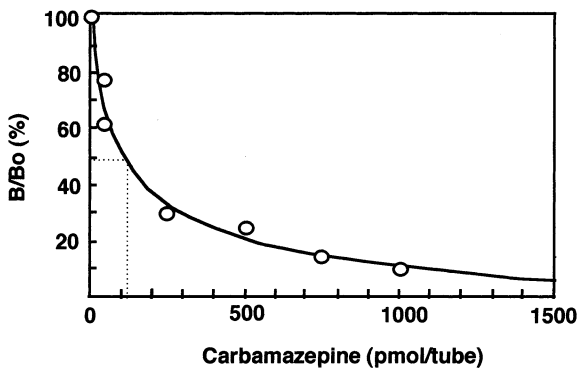

Fig. 6. Standard curve of carbamazepine by CMIA obtained in the presence of 30 pmol of $Co_2(CO)_6$-carba.

munisation with the three immunogens displayed in Fig. 2 [13,16,17]. Antisera were characterised by establishment of their dilution curves. A typical curve is shown in Fig. 5. It was obtained by incubating 30 pmol of the carbamazepine tracer in the presence of increasing dilutions of anti-carbamazepine antiserum. The free fractions of tracer were separated by extraction with isopropyl ether which was evaporated under vacuum. Quantities of free tracer were eventually measured by recording their IR spectrum in carbon tetrachloride and measurement of the peak height at 2056 cm^{-1}. The quantities of bound tracer B were calculated by subtraction of the peak height of the control tube. In the case shown here, the titre value, by definition the amount of antiserum needed to complex 50% of the tracer, was found to be 800 [17].

This titre value reflects the quantity of antibody present in the antiserum, but does not give any indication of the ability of using these antibodies to assay the analyte. This was provided by the standard curve such as the one displayed in Fig. 6. This curve was obtained by incubating a fixed amount of antibody, equal to the titre value, in the presence of 30 pmol of $Co_2(CO)_6$-carba and increasing quantities of the analyte, in this case carbamazepine. Afterwards, for each sample, the percent bound fraction B/B_0 was measured, with B representing the quantity of bound tracer and B_0 the quantity of tracer bound in the absence of carbamazepine. These B/B_0 values were measured with standard errors ranging between 2 and 3.6% [18]. For high quantities of carbamazepine, the B/B_0 values were close to zero, which shows good sensitivity for the assay. On the other hand, a B/B_0 value of 50% was obtained for a quantity of carbamazepine in the 100 pmole range, which shows good sensitivity for the assay. Based on the therapeutic range of carbamazepine (7–42 μM), this quantity corresponds to the amount of drug typically contained in few microliters of the serum of treated patients.

We also developed the mono-CMIA of phenobarbital [16] and DPH [13], the two other antiepileptic drugs. We extended this method to the assay of a steroidal hormone, cortisol [19], which is an endogenous substance present in the blood at levels approximately 100 times lower than the antiepileptic drugs and, more recently, to the assay of the pesticide atrazine [20].

After this preliminary work, the advantages of the CMIA method became clear: (1) absence of non-specific signal, perhaps the primary advantage of the technique, owing to the totally artificial nature of these labels; (2) sensitivity of the technique comparable to that of other immunological methods in the drug analysis; (3) short data acquisition time (1 min); (4) possibility of synthesising a tracer for any hapten, simply by adapting syntheses that were developed for the preparation of the immunogens; and (5) suitability of the method for use in a simultaneous multi-immunoassay (multi-CMIA) of several substances, thanks to the fact that each complex displays different bands.

4. Implementation of multi-CMIA

There has always been considerable interest in simultaneous multi-immunoassays, which would address well-established needs such as therapeutic drug monitoring of patients on multiple medications, thyroid testing, or screening for infectious agents. However, the multi-immunoassay field is still not well developed, since it appears difficult to find tracers emitting signals that can be analysed simultaneously. In fact, with radioactive labels or enzymes it has not been possible to analyse more than two compounds simultaneously [21–23], and only with fluorescent tracers (lanthanide chelates) has it been possible to assay simultaneously mixtures of three or four analytes [24]. As a number of authors have pointed out, multi-immunoassay is one of the current challenges in the immunoassay field [25,26] and we believe that CMIA could play a major role in this area.

In developing a double immunoassay, it was firstly necessary to establish the simultaneous quantitation of two tracers. In the simplest case, such as for mixtures of carbamazepine and DPH tracers, the IR spectral peaks did not overlap (see Fig. 4). Therefore, quantitation was readily done by measurement of the peak height of the two analytical bands at 2056 and 1971 cm^{-1}. For the two other mixtures of two tracers (phenobarbital + DPH and carbamazepine + phenobarbital tracers), the analytical band of one of the tracers was unobscured (phenobarbital tracer in the first case, carbamazepine tracer in the second one). Therefore, direct quantitation was performed by measuring the unobscured peaks, at 2032 and at 2056 cm^{-1} for these two tracers, respectively. In both cases, the two other tracers exhibited partial to complete overlap of their analytical bands. This required quantitation of the remaining two tracers to be carried out by a stepwise calculation, subtracting the contribution that the $Mn(CO)_3$ group made to the absorbance at 1971 cm^{-1} and that made by the $Co_2(CO)_6$ group to the absorbance at 2032 cm^{-1}. Eventually, the simultaneous quantitative analysis of any combination of two metal carbonyl tracers could be achieved in this manner with less than 5% error.

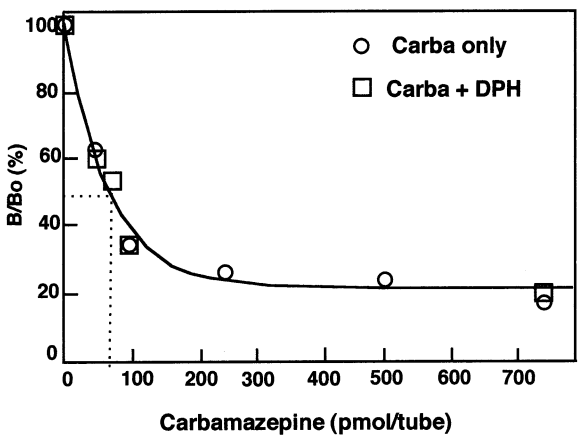

Fig. 7. Standard curve of carbamazepine in mono- and double-CMIA (mixture of carbamazepine and DPH).

Antiserum dilution curves were obtained for anti-carbamazepine and anti-DPH both separately and simultaneously by a double assay. Curves were found to be perfectly superimposable and both individual and double CMIA gave the same antiserum titre values, indicating that there was no cross-reaction between the two antibodies.

Standard curves for carbamazepine and DPH were obtained both separately and in the double immunoassay format (Fig. 7). Again both standard curves for carbamazepine could be superimposed, confirming the feasibility of the double immunoassay [18].

The next step was to proceed to the simultaneous immunoassay of the three antiepileptic drugs, carbamazepine, phenobarbital and DPH. For this, it was necessary to quantify the three tracers from spectra such as the one shown in Fig. 8. In this mixture, the analytical band of $Co_2(CO)_6$-carba remained independent while the analytical band of $Mn(CO)_3$-pheno was completely obscured under the 2027 cm^{-1} $Co_2(CO)_6$-carba band and the analytical band of $Cr(CO)_3$-DPH was partially overlapped by the 1950 cm^{-1} $Mn(CO)_3$-pheno band. Therefore, quantitative analysis required the use of multivariate analytical methods, such as K-matrix or partial-least-squares algorithms [27]. Wit the use of these methods, we now had access to the triple immunoassay of the three antiepileptic drugs [28].

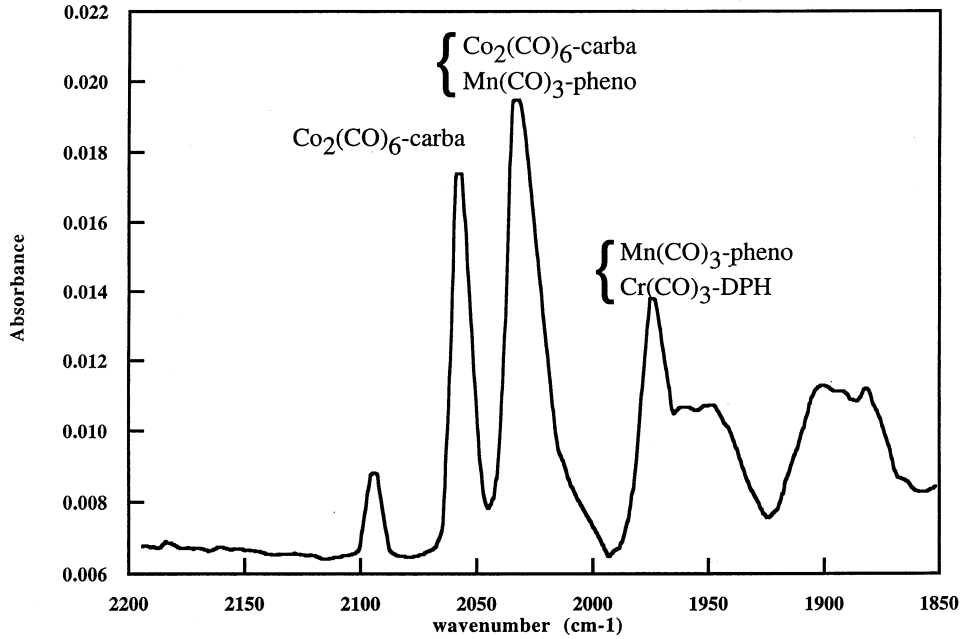

Fig. 8. IR spectrum (20 scans, 4 cm^{-1} resolution) of a mixture of $Co_2(CO)_6$-Carba, $Mn(CO)_3$-Pheno, $Cr(CO)_3$-DPH in solution in chloroform recorded on a MB100 FT-spectrometer (Bomem) equipped with an InSb detector and a light-pipe cell.

Thus, the CMIA method has been definitely shown to have a valid role to play in the field of simultaneous multi-immunoassays.

5. Conclusions and perspectives

We are now working towards two crucial goals in the perspective of development of CMIA in clinical analysis. The first goal is to increase the intensity of the infrared signal. The second goal is to simplify the separation step of the free and bound fractions. To achieve these goals, we are currently working on using metal carbonyl complexes to label proteins. This type of labelling would provide increased assay sensitivity by increasing the number of organometallic fragments attached for each protein molecule, and it has the added advantage of leading us to develop immunoassays for proteins, which is a large segment of the immunoassay market. We are also developing a solid-phase immunoassay, based on gold surfaces where one of the immunological reagents could be immobilised and which should be compatible with the infrared detection step.

Acknowledgements

Financial support was provided by France–Quebec exchanges, Conseil Régional de Bourgogne, DRET and CNRS. We wish to thank Barbara McGlinchey for her assistance in translating the manuscript.

References

[1] J.P. Gosling, Clin. Chem. 36 (1990) 1408–1427.
[2] S.V. Rahavendran, H.T. Karnes, Pharm. Res. 10 (1993) 328–334.
[3] G. Jaouen, A. Vessières, I.S. Butler, Acc. Chem. Res. 26 (1993) 361–369.
[4] T. Hirschfeld, in: J.R. Ferraro, L.J. Basik (Eds.), Fourier transform infrared spectroscopy. Applications to chemical systems, Academic Press, New York, 1979.
[5] H.M. Heise, R. Marbach, T.L. Koschinsky, F.A. Gries, Appl. Spectrosc. 48 (1994) 85–95.
[6] M. Volmer, A. Bolck, B.G. Wolthers, A.J. de Ruiter, D.A. Doornbos, W. van der Slik, Clin. Chem. 39 (1993) 948–954.
[7] M. Salmain, A. Vessières, G. Jaouen, I.S. Butler, Anal. Chem. 63 (1991) 2323–2329.

[8] A. Vessières, G. Jaouen, M. Salmain, I.S. Butler, Appl. Spectrosc. 44 (1990) 1092–1094.
[9] G.O. Spessard, G.L. Miessler, Organometallic Chemistry, Prentice-Hall, Upper Saddle River, 1997.
[10] A. Fazio, E. Perucca, F. Pisani, J. Liq. Chrom. 13 (1990) 3711–3740.
[11] M. Salmain, A. Vessières, I.S. Butler, G. Jaouen, Bioconjug. Chem. 2 (1991) 13–15.
[12] I. Lavastre, J. Besançon, P. Brossier, C. Moïse, Appl. Organomet. Chem. 4 (1990) 9–17.
[13] A. Varenne, A. Vessières, P. Brossier, G. Jaouen, Res. Commun. Chem. Pathol. Pharmacol. 84 (1994) 81–92.
[14] G. Bör, J. Organomet. Chem. 94 (1975) 181–188.
[15] F.A. Cotton, A.D. Liehr, G. Wilkinson, J. Inorg. Nucl. Chem. 1 (1955) 175–186.
[16] M. Salmain, A. Vessières, P. Brossier, I.S. Butler, G. Jaouen, J. Immunol. Methods 148 (1992) 65–75.
[17] A. Varenne, A. Vessières, M. Salmain, P. Brossier, G. Jaouen, J. Immunol. Methods 186 (1995) 195–204.
[18] A. Varenne, A. Vessières, M. Salmain, S. Durand, P. Brossier, G. Jaouen, Anal. Biochem. 242 (1996) 172–179.
[19] V. Philomin, A. Vessières, G. Jaouen, J. Immunol. Methods 171 (1994) 201–210.
[20] G. Patole, A. Vessières, S. Ben Rejeb, F. Le Goffic, G. Jaouen (unpublished results).
[21] F.H. Wians, J. Dev, M.M. Powell, J.I. Heald, Clin. Chem. 32 (1986) 887–890.
[22] K.J. Dean, S.G. Thompson, J.F. Burd, R.T. Buckler, Clin. Chem. 29 (1983) 1051–1056.
[23] C. Blake, M.N. Al Bassam, B.J. Gould, Clin. Chem. 28 (1982) 1469–1473.
[24] Y.Y. Xu, K. Pettersson, K. Blomberg, I.A. Hemmilä, H. Likola, T. Lövgren, Clin. Chem. 38 (1992) 2038–2043.
[25] L.J. Kricka, Clin. Chem. 38 (1992) 327–328.
[26] R. Ekins, F.W. Chu, Clin. Chem. 39 (1993) 369–370.
[27] A. Varenne, A. Vessières, M. Salmain, G. Jaouen, Appl. Spectrosc. 52 (1998) 1383–1390.
[28] M. Salmain, A. Vessiéres, A. Varenne, P. Brossier, G. Jaouen, J. Organometal. Chem. (in press).