Nanoscale interaction for higher efficiency of contrast media

(Settore scientifico-disciplinare CHIM/04)
ABSTRACT

Iopamidol, Iomeprol and Iopromide are Non-ionic Iodinated Contrast Media (NICMs) are used as concentrated solutions in x-Ray diagnostics as angiography and urography. The analysis of the current literature knowledge on NICMs shows an clear abundance of clinical diagnosis data but a lack of information on their physico-chemical properties. The success of these molecules in diagnosis is due to a combination of their properties, but a clarification of the role of structural determinants affecting the processes in concentrated solution is necessary. Thus a study of the molecular details may shed light on the differences in physico-chemical behavior. The concentrated solutions of NICMs are characterize by low viscosity and osmolality values due to the self-assembling of the system that generates soluble nano-structured aggregates in aqueous solution. Standing these considerations, the research work focused on spectroscopic, thermodynamics and MD simulation techniques to probe the association phenomenon, as a function of concentration and temperature. The interplay of the intermolecular interactions are the main reason for the stability of the concentrated solution of NICMs. Non-ionic iodinated contrast media have quite a simple molecular structure, but they show a complex behaviour due to the atropisomerism phenomenon. The coexistence of several structural isomers (atropisomers) in solution is at the basis of the different geometries of solute-solute interactions. Thus, the first study whas been the molecular characterization of these molecules by using NMR spectroscopy to probe the conformational equilibria in terms of conformer population in solution. Thermodynamic approaches provided a classification of Iopamidol, Iomeprol and Iopromide according to their thermodynamics behaviour in terms of hydrophilic and hydrophobic interactions with water molecules. In parallel, MD simulations data were carried out to provide information about hydration shell (which were compared with experimental data from literature) and aggregation process. Similarly, the association was probed by both NMR and experimental thermodynamic data. To have more information on the nature of intermolecular...
interactions and atropisomerism phenomenon the solids of Iomeprol and Iopamidol (either or glasses and crystals) were analyzed by spectroscopic, calorimetric and diffractometric techniques that shown agreement in terms of intermolecular interactions among side chains. Furthermore, solid-solid transitions were detected as a function of temperature. By collecting experimental data by isothermal and scanning calorimetry, thermodynamic properties, molecular dynamics simulations and especially by several spectroscopics methods a coherent description of the structure and dynamics of NICMs has been achieved. These results provide new knowledge on thier physico-chemical properties and allow us to interpret some unclear phenomena.
Iopamidolo, Iomeprolo e Iopromide: Mezzi di Contrasto Iodinati Non-ionici (MCIN) usati in urografia e angiografia come soluzioni concentrate. I dati relativi ai MCIN presenti in letteratura mostrano un’abbondanza di informazioni cliniche ma una carenza di dati riguardanti le loro caratteristiche chimico-fisiche. L’efficacia di questi composti in ambito medico è dovuta alla combinazione di tali proprietà. Risulta quindi necessario uno studio relativo ai dettagli molecolari per chiarire i contributi di ogni gruppo funzionale del sistema che determinano le differenze in termini di comportamento chimico-fisico. Le soluzioni concentrate di MCIN sono caratterizzate da bassi valori di viscosità e osmolalità dovuti all’autoassemblamento del sistema che genera aggregati nanostrutturati solubili in soluzione acquosa. Da queste considerazioni, questo lavoro di ricerca si è focalizzata su tecniche spettroscopiche, termodinamiche e di simulazioni di dinamica molecolare per indagare il fenomeno dell’associazione, sia in funzione della temperatura che della concentrazione, relazionato alle interazioni intermolecolari che spesso sono la principale causa della stabilità delle soluzioni concentrate di MCIN. Questi composti possiedono una struttura molecolare semplice, ma sono sistemi complessi in quanto soggetti all’atropisomerismo che causa la coesistenza di isomeri strutturali (atropisomeri) in soluzione, quindi di diverse geometrie di interazione soluto-soluto. Il primo approccio sperimentale è stata la caratterizzazione molecolare attraverso la spettroscopia NMR per determinare gli equilibri conformazionali in termini di percentuali di popolazione in soluzione. Studi termodinamici hanno permesso di classificare Iopamidolo, Iomeprolo e Iopromide in base alle loro caratteristiche idrofiliche ed idrofobiche nei confronti delle molecole di acqua. Parallelamente sono state realizzate simulazioni di dinamica molecolare per ottenere informazioni riguardo la sfera di idratazione (confrontate con i dati termodinamici da letteratura) e sul processo di associazione che è stato studiato in funzione della temperatura sia con la spettroscopia NMR che con quella Brillouin. Ulteriori informazioni sulle interazioni intermolecolari e sull’atropisomerismo sono state ottenute analizzando anche lo stato solido dello Iopamidolo e dello Iomeprolo (sia sui vetri che sui
cristalli) tramite tecniche spettroscopiche, clorimetriche e diffrattometriche i cui risultati hanno mostrato analogie in termini di interazioni intermolecolari fra le catene laterali. Inoltre, studi in funzione della temperatura hanno mostrato alcune transizioni solido-solido. L’accumulo di dati sperimentali relativi alla calorimetria di diluizione, calorimetria differenziale a scansione, letteratura, traiettorie di dinamica molecolare e spettroscopie, ha permesso di estendere il quadro generale delle proprietà chimico-fisiche dei MCIN.
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1. INTRODUCTION

1.1 IODINATED CONTRAST MEDIA, BRIEF HISTORY

The initial use of iodinated contrast media in medicine was in early 1900, when Marcel Guerbet, (Professor of Toxicology at the School of Pharmacy in Paris) discovered that an organic compound containing iodine (Liopidol) could be used as contrast medium in radiology because it absorbed X-rays\[^1\].

Iodine is a radio-opaque element and is used to differentiate the soft tissues of the body as veins and arteries which have the same ability to absorb the radiation and thus they would not be distinguishable during radiographic analysis\[^2\]. The first X-ray examination, which was performed for the spinal cord, dates back to 1921 and increased the interest to these organic compounds. The studies focused initially on the water solubility and toxicity. In 1928, a sodium salt of 5-iodo-2-piridone-N-acetic acid was synthesized. It was the first iodinated organic contrast medium for intravenous use. Wallingford, in 1950, discovered a derivative of benzoic acid: the sodium acetrizoate which was the first triiodinated compound. All contrast agents that were synthesized after the sodium acetrizoate contained always three iodine, in position 2,4,6 of the aromatic ring, atoms, while in position 1,3,5 suitable functional groups were able to modulate both solubility and toxicity. Wallingford discovered that an acetylated amino moiety in position 3 and 5 reduced the toxicity of the compound.

Before 1969 all contrast media were ionic monomer characterized by high solubility and high osmolality. These compounds, however, were responsible for severe side effects in patients. This fact induced to study and to synthetize new non-ionic drugs characterized by low osmolality. In 1969 Torsten, a Swedish radiologist, proposed the first non-ionic contrast medium. Since then, all the molecules of iodinated contrast media were monomeric, non-ionic with low osmolality. More recent studies aim at synthesizing non-ionic and dimeric type iodinated contrast agents characterized by osmolality values close to that of blood (280-295 mOsm/kg\text{water}). In this thesis work monomeric Non-Ionic Contrast Media, NICMs, as Iopamidol, Iomeprol and Iopromide were studied.
1.2 TYPES OF IODINATED CONTRAST MEDIA

Chemical point of view

Iodinated contrast media (ICMs) are part of a category called Positive contrast media and are characterized by a high mass number. ICMs media can be divided in classes from 1 to 6 defined as:

$$class = \frac{N_1}{N_{MS}}$$  \hspace{1cm} (1.1)

where $N_1$ is the number of iodine atoms and $N_{MS}$ is the number of particles released in solution after dissolution. For Ionic Iodinated Contrast Media (IICMs) $N_{MS} = 2$; one is the anion and one is the cation, while, for NICMs $N_{MS} = 1$.

The ionicity of these molecules is due to the presence of benzene carboxylic group on the ring associated with a non-radio opaque cation (calcium, sodium or methylglutamine) in aqueous solution and is mainly due to the presence of carboxyl moiety, directly attached to the aromatic ring.

The ICMs can be distinguished in monomeric and dimeric compounds, and, furthermore, in ionic and non-ionic molecules.

The ionic monomers dissociate in anion and cation. The consequence is an increase of the osmolality value that becomes double. This is a problem for patient safety. Considering the ratio number of iodine atoms/number of particles in solution in this system is 3:2.

The non-ionic monomers show the benzene ring differently substituted in position 1, 3, 5 by side chains to ensure adequate solubility. These compounds do not dissociate in solution and their atoms of iodine:particle ratio is 3:1. The osmolality\cite{3,4} is reduced by half compared to the ionic monomers and this is an advantage because an efficient iodinated contrast media should be isosmolar as the blood.

The ionic dimers are composed by two monomers, one is a ionic and the other is non-ionic monomer and are bonded through covalent bond. The atomic
ratio of iodine:particles is 6:2. The image quality is greatly increased compared to previous compounds.

The non-ionic dimers consist of two non-ionic monomers, bonded through covalent bond, and the iodine atoms: particles ratio value is 6:1.

As for the contrast, image quality is almost equal to that of ionic monomers; indeed, in terms of image quality (contrast) there are no differences between ionic and non-ionic monomer compound. Furthermore, the ionic contrast media cause an high incidence of side effects compared to non-ionic contrast media. Thus, nowaday, non-ionic contrast media are mainly marketed and proposed because they show a low osmolality and so toxicity. The side effects are due to the perturbation of the electrical potential of cell membranes\(^5\).

**Clinical point of view**

Iodinated contrast media may also be classified in terms of clinical point of view considering parameters as viscosity and osmolality\(^6\) as shown in table 1.1.

**H.O.C.M.** (High osmolar Contrast Media) have a high osmolality (1400-2016 mOsm / kg of water). The incidence of side effects in patients treated by this type of contrast media is very high.

**L.O.C.M.** (Low osmolar Contrast Media) have a low osmolality that can varies between 600 and 884 mOsm / kg of water. These values of osmolality are closer to that of blood (280-295 mOsm / kg water) and their production and marketing are preferred.

**L.V.C.M.** (Low Viscosity Contrast Media) have a low viscosity whose values are between 20.9 and 26.6 mPa.s

**H.V.C.M.** (High Viscosity Contrast Media) have a high viscosity whose values are between 15.7 and 20.4 mPa.s.
Introduction

### Table 1.1: Classification of iodinated contrast media

<table>
<thead>
<tr>
<th>CLASS</th>
<th>CHEMICAL NAME</th>
<th>COMMERCIAL NAME</th>
<th>OSMOLALITY (mOsm/kg water)</th>
<th>VISCOSITY at 20 °C (mPa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H.O.C.M.</td>
<td>Ionic monomers</td>
<td>Diatrizoate</td>
<td>Hypaque</td>
<td>2016</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RenoCal-76</td>
<td>1870</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MD-76</td>
<td>1551</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Iotalamate</td>
<td>1400</td>
</tr>
<tr>
<td>H.V.C.M.</td>
<td>Non-ionic dimers</td>
<td>Iodixanol</td>
<td>Visipaque 320</td>
<td>290</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Iopromide</td>
<td>774</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Iopamidol</td>
<td>796</td>
</tr>
<tr>
<td>L.O.C.M.</td>
<td>Non-ionic monomers</td>
<td>Iohexol</td>
<td>Omnipaque 350</td>
<td>844</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Isovue 370</td>
<td>792</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Optiray 350</td>
<td>695</td>
</tr>
<tr>
<td></td>
<td>Ionic dimers</td>
<td></td>
<td>Hexabrix 320</td>
<td>600</td>
</tr>
</tbody>
</table>

A good combination of viscosity and osmolality could improve the quality of iodinated contrast media in terms of side effects and stability of the solutions.

The recent science research has focused on the synthesis of iodinated contrast media that can be isosmolar to the blood, while limiting the maximum increase in viscosity.

### Iodine contrast

Iodine has three fundamental features to get a good radiological contrast medium:

- Its atomic number is 53 with an electronic density seven time more than the carbon. It allows good contrast because the results show images very well defined in which the soft tissues of the body can be efficiently examinated.

- It is involved in a covalent bond with the aromatic ring and the mesomeric effect stabilize the system (C-I, 0.20 nm). The non-ionic iodine is chemically stable and does not generate side effects as ionic iodine.

- The body tollerates the benzene tri-iodo substituted. The iodine concentration in the solution is the most important parameter
in radiology and it is defined as the iodine weight contained in a volume unit (mgI/mL).

An high concentration of iodine allows low dosage of radiations because the contrast is improved\cite{3}.

Contrast is defined as:

\[
C = \frac{g_0}{g_b}
\]

\[1.2\]

\(g_0\) is the grey level in the main subject and \(g_b\) is the grey level in surrounding background. Various x-Ray examination are performed according to the body zones to analyze and each technique requires a different iodine concentration in the blood. The consequence is the commercialization of iodinated contrast media solutions at different concentrations.

1.3 CLINICAL APPLICATION

Iodinated contrast media (ICM) are used in both radiology and imaging diagnostic in order to increase, or create, the necessary contrast between soft tissues of the body, organs and blood vessels. In radiology only body zones that interact selectively with X-rays can provide a defined radiographic image. If an organ absorbs little radiation, or absorbs the same manner as the adjacent organs, it will not be visible in the image or will be very attenuated. This is the case of the stomach, liver, kidneys and many other abdominal organs.

The ICMs are used in various techniques as angiography (DSA), computed tomography and urography\cite{7}.

The DSA (Intraarterial Digital Subtraction Angiography) is a radiological examination that allows to study the morphology and the course the blood vessels in different parts of the body, reporting any changes (vascular constrictions, occlusion or dilatation).

Using very thin catheters blood vessels and their branches can be reached and the injection is very localized. This fact allows to realize a selective study.
**Table 1.2**: Iodinated contrast media and their clinical application fields.

<table>
<thead>
<tr>
<th>Contrast Media</th>
<th>Iodinated CM</th>
<th>Non-ionic dimers</th>
<th>Ionic dimers</th>
<th>Non-ionic monomers</th>
<th>Ionic monomers</th>
<th>Insoluble CM in H₂O</th>
<th>Oily CM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urography and Angiography</td>
<td>Iodecol</td>
<td>Iotrolan</td>
<td>Ioxaglate</td>
<td>Iodipamate</td>
<td>Iopamidol</td>
<td>Iopodate</td>
<td></td>
</tr>
<tr>
<td>Mielography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ultrasound CM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Air, CO²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>Oily CM</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodinated CM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoic acid derivatives</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insoluble CM in H₂O</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diatrizoate</td>
<td>Metrizamide</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iopamidol</td>
<td>Lopronate</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Iopodate</td>
<td></td>
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</tbody>
</table>
Urography is performed to study the upper and lower urinary tract by intravenous injection of iodinated contrast media. Computed tomography (CT) is a diagnostic method that allows to obtain images of cross sections of the human body by statistical-mathematical elaboration of x-ray absorption data (computerized). In the diagnostic CT examinations the intravenous injections are required. The application fields of iodinated contrast media are summarized in the table 1.2.

1.4 MOLECULAR STRUCTURE OF ICMs

Common moiety

The common molecular part of ICM currently available for intravenous injection comprises a tri-iodide benzene ring substituted. The iodine atoms are bonded in position 2, 4 and 6 of aromatic ring. While, in position 1, 3 and 5, three aliphatic chains are linked.

The nature of the side chains generates the main differences among ICMs in terms of molecular structure and physico-chemical properties. Each side chains are connected to the common aromatic moiety through the amidic bonds. In the Figure 1.1 the common moiety of ICMs is shown.

![Generic molecular structure of ICMs.](image)

where $R_1$, $R_2$ and $R_3$ are aliphatic chains opportunely functionalized by hydrophylic functional groups to improve the solubility and to decrease the
toxicity. The solubility of ICMs is very important because they have to act in a physiological environment.

This molecular structure is advantageous because allows to get compounds characterized by an high content of iodine. So, a tri-iodinated compound of this type shows an high effective iodine concentration\(^8\) that allow to get better results.

In angiography and urography the NICMs used are secreted by the kidneys and their good elimination is due to the hydrophylic moieties linked to the side chains\(^9,10\).

**Synthesis**

The most important step in the synthesis of ICMs is the introduction of the iodine into the molecule. Iodine is introduced either through substitution or by addition reactions. The benzene ring can be iodinated by a number of methods via the intermediate I\(^+\), which will react with position of the benzene ring activated by neighbouring electronegative groups. If the electronegativity is not enough, then the number of iodine atoms introduced into the benzene ring is reduced, less than three. Normally, amine groups are used for activation. The active iodine ion (I\(^+\)) is generated in aqueous solution from ClI. The resulting tri-iodinated compounds are usually less water soluble than their precursors and precipitate. Purification of the intermediates is easily performed. Following acylation of the amino groups, the synthesis of ionic iodinated contrast media is complete. The acylation of the amino moieties significantly increase their electronegativity and thereby stabilize the C-I bonds (decreasing of toxicity). As a typical reaction scheme for an ionic monomer, the synthesis pathway for diatrizoate is given in figure 1.2.

The synthesis of Non-Ionic Contrast Media (NICM) is much more complicated. Due to the higher aqueous solubility of the intermediates, purification processes have to be more sophisticated than for the ionic compounds. Non-ionic compounds require significantly more synthesis steps, particularly for some unsymmetrical types. General features of the synthesis are identical to those used for ionic contrast media: hydrogenation of nitro groups
(Figure 1.2) but, for NICMs, amidation of carboxyl groups via carboxyl chlorides becomes necessary (figure 1.3). Furthermore, NICMs do not dissociate during the solubilization process and the consequence is a non-increase of osmolality values in aqueous solution. Infact, osmotic coefficient is a colligative property which depends from the number of particles released in the solution.

![Chemical structures](image)

**Figure 1.2**: synthetic pathway to ionic iodinated contrast media, reaction scheme for Diatrizoate.

![Chemical structures](image)

**Figure 1.3**: generic amidation of carboxyl group to synthetize a NICM.
Major efforts, however, have to be directed towards sophisticated purification procedures, since simple recrystallization is often no longer effective. Priebe et al.\cite{11} have described the synthesis and purification of Iodixanol (figure 1.4) and its physical and toxicological properties, and analytical and spectroscopic data.

Figure 1.4: molecular structure of Iodixanol, a non-ionic dimer.

Examples of other molecular structure of non-ionic contrast media as monomers are shown in the follow image, figure 1.5.
**Introduction**

**Figure 1.5**: examples of NICM molecular structures.

**Iopamidol, Iomeprol and Iopromide**

Iopamidol, Iomeprol and Iopromide are the three NICMs studied in this thesis work. In the figure 1.6 their molecular structures are shown.

**Figure 1.6**: molecular structures of Iopamidol, Iomeprol and Iopromide. The stereo centers are labeled green.

The differences among these three NICMs, in terms of molecular structure, are due to the small change in position of some moieties as methyl group and to the stereoisomerism of the side chains. A brief comment on the molecular structure of Iopamidol, Iomeprol and Iopromide is made below in terms of iodine content and molecular weight.
Iopamidol

The molecular structure of Iopamidol is shown in the figure 1.6. Its IUPAC name is N,N’-bis-(1,3-dihydroxy-2-propyl)-5-L-lactoylamino-2,4,6-triiodoisophthalamide\(^{12}\).

Iopamidol is an iodinated contrast media that belongs to the class of non-ionic monomers characterized by low viscosity (argument explained in the next paragraph)

- Molecular weight: 777.1 g/mol
- Iodine content: 49 % w/w

In its molecular structure, attached to the nitrogen bonded in position 1, a lactate moiety is bonded trough amidic bond. In the lactate group the carbon C 11, adjacent to the carbonyl moiety, is a chiral center. Two enantiomers are possible named \(D\) and \(L\) respectively. To stabilize the Iopamidol solutions, an amount of excipients is added. They consist of trometamol and sodium calcium edeteate. On the market the Iopamidol solutions are commercialized as \(L\) enantiomer only at 99%. Furthermore, in the table 1.3 the commercial names of Iopamidol, marketed by different pharmaceutical company, are shown.

Table 1.3: commercial names of Iopamidol.

<table>
<thead>
<tr>
<th>COMMERCIAL NAME</th>
<th>PHARMACEUTICAL COMPANY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iopamiro®</td>
<td>Bracco</td>
</tr>
<tr>
<td>Isovue®</td>
<td>Squibb</td>
</tr>
<tr>
<td>Niopam®</td>
<td>Merk, UK</td>
</tr>
<tr>
<td>Solutrast®</td>
<td>Byk Gulden</td>
</tr>
<tr>
<td>Iopamiron®</td>
<td>Schering</td>
</tr>
</tbody>
</table>
Iomeprol

Iomeprol molecular structure is shown in the figure 1.6. Its IUPAC name is N,N’-bis-(2,3-dihydroxypropyl)-5-[(hydroxyacetyl)methylamino]-2,4,6-tri-iodo-1,3-benzenedicarboxamide[4].

It is an iodinated contrast media that belongs to the class of non-ionic monomers characterized by, as Iopamidol, low viscosity.

- Molecular weight: 777.1 g/mol
- Iodine content: 49 % w/w

The side chains attached in position 3 and 5 through amidic bond consist of two serinolic moieties. This moiety shows a chiral center. So, in Iomeprol two chiral centers are present and potentially four diastereoisomers can be exist: RR, SS, SR and RS.

Iomeprol is obtained by a synthesis that is particularly friendly to the environment. Iomeprol has allowed pharmaceutical formulations of iodinated contrast media, Iomeprol injection (Iomeron®, Imeron®), which have the lowest osmolalities of all currently available contrast media that are based on tri-iodinated non-ionic monomers. These formulations are offered as ready-for-use solutions. Iomeprol injection also has the lowest viscosity. These properties combined with an ability to form highly concentrated solutions that have no tendency to crystallize out have allowed the formulation of Iomeprol at 400 mg/l/mL, a concentration not previously practical with non-ionic iodinated contrast media. The combination of lowest osmolality, lowest viscosity, high solubility and the absence of EDTA places Iomeprol in a unique position and guarantees minimal side effects attributable to physico-chemical properties and pharmaceutical excipients[4]. Iomeprol is marketed under the name “Iomeron®” at various iodine content.

Iopromide

Molecular structure of Iopromide is shown in the figure 1.6. Its IUPAC name is N,N’-bis-(2,3-dihydroxypropyl)-2-4-6-tri-iodo-5-[(2-methoxyacetyl) amino]-N’-methyl-benzene-1,3-dicarboxyamide[13].
Introduction

- Molecular weight: 791.1 g/mol
- Iodine content: 48 % w/w

The side chains attached in positions 3 and 5 are the same than Iomeprol and this implies two chiral centers. While the side chain in position 1 does not show further chiral centers. So, commercial Iopromide is a mixture of diastereoisomers.

Iopromide is marketed by Schering company as aqueous solution at various concentrations (Table 1.4).

**Table 1.4:** Iopromide, commercial name is Ultravist, marketed by Schering.

<table>
<thead>
<tr>
<th>Commercial name</th>
<th>mg iopromide/mL solution</th>
<th>Radiographic technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultravist 150</td>
<td>312</td>
<td>Angiography (D.S.A.)</td>
</tr>
<tr>
<td>Ultravist 240</td>
<td>499</td>
<td>Cranial computed tomography (CT), arteriography, venography, urography, analysis of the subarachnoid cavity and other cavity</td>
</tr>
<tr>
<td>Ultravist 300</td>
<td>623</td>
<td>The same applications of Ultravist 240 with the exception of subarachnoid cavity</td>
</tr>
<tr>
<td>Ultravist 370</td>
<td>769</td>
<td>The same applications of Ultravist 300 with a preferential use for angiography</td>
</tr>
</tbody>
</table>

The similarity in the molecular structure among NICMs is evident. Optimization of physico-chemical properties is given by understanding the role that the side chains play in aqueous solution.

The side chains, thus, are responsible for the elimination of the NICMs from the body.
1.5 PHYSICO-CHEMICAL PROPERTIES

Premise

The history of iodinated contrast media, illustrated in the paragraph 1.1, allows to say that the choice of the functional groups involved in the iodinated contrast media is planned and targeted observing the essential elements in the evolution of these systems.

Scientific research has involved two main branches of science:

- Medicine
- Chemistry

The need for medicine to have NICMs mainly characterized by a high content of iodine induced the chemical research to found synthetic strategies to synthetize multi-iodinated organic compounds. The results of the research led to formulate some organic synthesis to satisfy the demands of medicine.

The most of the chemical strategy show the common heart of NICMs that is, as mentioned above, the tri-iodo substituted aromatic ring. So, to sum up, the scientific research has been focused on two aspects of these systems: to synthesize organic molecules with a high content of iodine (chemistry) and to decrease their toxicity (medicine). The overall results of NICM solutions curried out untill now, which are published in literature, allow to say that NICM solutions are mixtures characterized by partly unexplored and especially not fully understood properties. A research study, for example, demonstrated that the Iopamidol influences the solvation of proteins in physiological enviroment\(^{[14]}\).

Defined the central "unit", the specific molecular structure reached is the results from optimization of some well defined properties: high solubility, low osmolality and low viscosity. To change the properties listed above is necessary to
change the side chains. The basis of solubility, osmolality and viscosity consist of interactions between water-NICM and NICM-NICM molecules.

To synthetize flexible and water-soluble oligomers of NICMs would be a method to optimize the solubility and osmolality, but side effects may be present and this method is prohibitive for changes in viscosity that is imparted to the solution. From these considerations, the study of intermolecular interactions was considered to be almost essential to understand, at least in part, their role in the self-assembly of NICMs process (in aqueous solution) which generates the nanostructured soluble aggregates. Therefore, this optimization of the global characteristics led the physical-chemistry to analyze these systems to provide an understanding of molecule-molecule and molecule-solvent interactions which are the basis of all the properties of the NICM solutions.

In other words, the concept of covalent polymerization (chemical bond) is substituted with the concept of non-covalent polymerization-aggregation (weak interactions that fluctuating).

This premise was necessary before introducing the description of some thermodynamic properties of the NICMs in question. In the following pages, therefore, will be discussed the essential elements of the properties defined above: osmolality, solubility and viscosity, while a description of the enthalpy property, studied in part in this thesis, is shown later.

**Osmolality**

The osmolality indicates the molal concentration \( \text{mol}_{\text{solute}}/\text{kg}_{\text{solvent}} \) of molecules osmotically active in the solution at certain concentration. The osmotic concentration, that is the osmolality, was defined by Robinson and Stokes\(^{[15]} \) as the \( \nu m \Phi \) multiplication, where \( \nu \) is the species number in the solution, \( m \) is the molality and \( \Phi \) is the osmotic molal coefficient (non-ideal measure of the solution).

For a non-ionic solute was possible, by elaborating the Gibbs-Duhem equation, to relate \( \Phi \) and \( \gamma \) (activity molal coefficient). Equation 1.3:
\[
\ln \gamma = (\Phi - 1) + \int_{0}^{m} (\Phi - 1) d \ln m \tag{1.3}
\]

The polynomial approach is the most analytical form used to express the osmotic coefficient: \(1 + a_1m + a_2m^2 + a_3m^3 + \ldots\) that is easy to integrate and to differentiate. At infinite dilution, the osmotic coefficient value tends to 1. This condition is implicitly given by the equation 1.3 that defines \(\gamma = 1\) when \(\Phi = 1\).

A value of \(\Phi\) very different than 1 implies strong deviations from diluted solution behaviour due to the non-negligible interactions between solute-solvent (\(\Phi > 1\)) and solute-solute (\(\Phi < 1\)) molecules.

In figure 1.7 the two cases, illustrated above, are shown. The osmotic coefficient trends are plotted for a very hydrate system (as sucrose or glucose) and for a very assembled system (as purine or caffeine).

![Figure 1.7: Osmotic coefficient trends as a function of concentration for a (1) hydrated molecules (2) assembled molecules.](image)

To understand osmolality concept in terms of deviation from ideal system, and so in chemical thermodynamic terms, is necessary to consider the original
definition (equation 1.4) of the osmotic molal coefficient ($\Phi$) reported to a solution:

$$\Phi = -\frac{1}{r} \ln a_i$$  

where $a_i$ is the activity of the solvent and $r = x_2/x_1$ ($x_1$: molar fraction of the solvent; $x_2$: molar fraction of the solute).

The activity of the solvent in a solution is defined as the ratio (equation 1.5) between the fugacity of the solvent vapor and that of the pure solvent (standard state):

$$a_i = \frac{f_i}{f_i^0}$$

Where $f_i^0$ is the fugacity of the pure solvent and $f_i$ is that of the solvent vapor above the solution. To emphasize the deviation from ideal solution, the activity coefficient of the solvent is used and is defined as (equation 1.6)

$$\gamma = \frac{f_i}{x_i f_i^0} = \frac{a_i}{x_i}$$

An advantage, by using the molal concentration, is the lack of the temperature into the equations.

The osmotic coefficient is a free energy “reduced”. So, the temperature dependence becomes implicit and is involved in the overview of the thermodynamic correlations: “first derivates” of the free energy.

Therefore, the osmolality of a solution and its change with temperature are a direct expression of both the interactions among the components of the solution (intensity and fraction expressed by excess free energy) and the enthalpy component of the interaction processes (intensity and fraction expressed by partial molar enthalpy).
Solubility

In mixed systems, at thermodynamic equilibrium, miscibility of the components could be not total and, in the diagram of composition/temperature, a curve appears and highlights the coexistence of two phases.

In a saturated solution the singularity of the curve is given by the equality of the chemical potential of both crystalline solid and the solute solvated in the saturated solution. The curve, composition/temperature, defines the enthalpy change of the dissolution of the crystalline solid in a saturated solution\textsuperscript{[16]}. In a real saturated solution some complications, due to the non-ideality behaviour of the system, are originated: the presence of crystalline polymorphic solids, the high viscosity which opposes the equilibration, the presence of various conformational states characterized by slow equilibration kinetics. A typical phase diagram of composition/temperature is shown in Figure 1.8 that are systems consisting of trehalose. Differences in solubility are evident for the two polymorphic crystalline phases in question that are thermodynamically defined. Moreover, the behavior of the glassy phase was studied at various composition.

![Figure 1.8: phase diagram composition/temperature of trehalose anhydrus, hydrated and the glassy transition.](image-url)
Also the solubility of a compound is known from a thermodynamic point of view, this fact does not imply the exclusion to carry out apparently contradictory data.

An example is given by one of the molecules studied in this work as Iopamidol[^7]. In literature its data of solubility[^3] of different polymorphic forms[^17] are published, but the graphical representations highlight the existence of other processes that alter the "simple" interpretation of a balance between crystalline phase and a saturated solution[^7].

At descriptive level it is obvious that the presence of hydrophilic functions in the iodinated contrast media increases their solubility and therefore this aspect is a subject much discussed and studied so far. By understanding and rationalizing of a way to get a complete solubilization and stabilization of these molecules is possible to prevent a possible phase separation of some less soluble polymorphs during clinical use.

As just mentioned above, the common molecular moiety of the NICMs is an triiodine substituted benzene which, without particular hydrophilic functionalizations, is insoluble in water. The commonly moieties, added to the side chains, consist of carbonyl, hydroxyl and polyhydroxylammide groups. In a system like this, the three iodine atoms generate more hindered rotations around the $\sigma$ bonds in position just illustrated above. This fact is due to the large atomic radius of the iodine atom which gives a high steric hindrance.

From a structural point of view the side chains hide hydrophobic parts of the NICM molecules increasing the solubility and decreasing the toxicity[^3].

The physical chemistry has focused, therefore, on ways to change these characteristics and on their quantification.

**Viscosity**

At the molecular level the viscosity is very well represented by the kinetic model of Eyring's theory that identifies the energy of vaporization (and therefore cohesion) as responsible for the value of the viscosity coefficient of a liquid. The viscosity expresses the deformation degree of the molecular interactions among the components when the fluid is subject to a dragging force.
In a solution, it expresses the degree of deformation of the molecular interactions between the components. Thus, there is an important "structural" correlation, from both theoretical and experimental points of view, between the viscosity and the thermodynamic properties of solutions. In general, iodinated contrast media with high-osmolality show low viscosity values and iodinated contrast media with low osmolality show high viscosity values.

The molecular volume is an important parameter because large molecular volumes give to the solution high viscosity values. In the cases studied in this work the viscosity values of the solutions are due mainly to the chemical structure type of the NICM. Only the side chains are able to move in the space (through the rotation of single bonds) and so only them can change the molecular structure because the aromatic system (the common moiety of all NICMs) is a rigid moiety. The NICM concentration influences the viscosity values. Viscosity increases with the "excluded volume" of the solute. This fact implies that the solutions of dimeric molecules are characterized by higher viscosity, while the monomeric systems show an opposite behavior. An increase of viscosity is also given by the rigidity degree increasing of the molecules. So, a solution of a flexible dimer is less viscous than a solution of rigid dimer.

The hindered rotation of the simple bonds in position 1, 3 and 5 gives to the system a certain degree of structural rigidity. A further consideration on the viscosity of iodinated contrast media is about the presence of hydroxyl groups in molecular structure which are responsible for the formation of both intra and inter-molecular hydrogen bonds.

The viscosity of NICMs is a relevant aspect because the solutions are injected intravenously and the media must reach, through the circulatory system, all the organs to be analyzed. Viscosity is the limiting factor because defines the highest usable iodine concentration in solution and, so, the best possible imaging.

In conclusion, the NICM solutions have a high viscosity, according to the maximum iodine content possible.
1.6 ATROPISOMERISM

Premise
In chemistry *atropisomerism* is a type of stereoisomery (configurational isomery) due to hindered rotations around single bonds (σ bonds generally). In other words atropisomerism is due to the pinned molecular conformations. This phenomenon is originated, generally, when close and large moieties are involved in the system. Two, or more molecular conformations, are separated by an amount of potential energy that usually can be overcome even at relatively low temperatures.

**Atropisomerism in the iodinated contrast media**
The common molecular structure of the iodinated contrast media shows hindered rotations around the single bond Aril-N and Aril-CO. This hindrance is due to the presence of iodine atoms. A general system of this type is shown in the figure 1.9.

![Figure 1.9: general scheme of hindered rotation in a NICM molecule.](image)

Several atropisomers of iodinated contrast media were studied by using chromatographic and spectroscopic techniques\textsuperscript{[1]}. An example is the study performed on Iotrolan (figure 1.10) that is a non-ionic iodinated dimeric contrast medium. Its isomery was studied by HPLC chromatography\textsuperscript{[1]}. 
The chromatographic analysis, at low resolution, of an aqueous solution of Iotrolan generates three peak groups (G1, G2 and G3) with a relative intensity of 68:4:28. At high resolution the G1 and G2 peaks, each of which, are divided into five different peaks characterized by the follow intensities: 1:4:6:4:1, while G3 peak generates seven peaks with relative intensity of 1:2:3:4:3:2:1. After separating the three peaks, G1, G2 and G3, their solutions were concentrated to obtain a concentration similar to that initial. These samples were thermically treated at 120 °C for 30 minutes and analyzed by HPLC again. The results shown that each isolated peak, after an heat treatment as that mentioned above, reaches the original peaks group pattern (original relative intensities).

Atropisomeric equilibria

The three iodine atoms that are covalently linked in position 2, 4 and 6 (figure 1.9) making the rotation around the bond of the remaining positions hindered and giving rise to atropisomerism. The presence of several conformational equilibria in solution, in this case aqueous solution, is the consequence of hindered rotation around single bonds. The atropisomeric equilibria that characterize the NICMs are shown in the figure 1.11.
Figure 1.11: from top to bottom syn/anti, SYN/ANTI and endo/exo equilibria.

- **syn/anti**: generated by the two possible relative orientations of the two carbonyl in positions 3 and 5 with respect to the aromatic ring plane;
- **SYN/ANTI**: generated by the two possible orientations of the anilido carbonyl with respect to the other carbonyls when the molecule is in syn form (this atropisomerism does not exist in the anti conformer);
• endo/exo: generated by the cis-trans equilibrium in the anilido moiety.
  (endo in case the carbonyl group is pointing inwards and exo in case it is
  pointing outwards);

In addition Z/E equilibria at the level of the amidic bonds are present in
the branches in positions 3 and 5. The atropisomerism phenomenon was studied
for Iopamidol by NMR\cite{18} and, in the case of Iomeprol, by HPLC\cite{4}, revealing the
presence of multiple conformers interconverting with energetic barriers ranging
from 74 KJ/mol to 83 KJ/mol\cite{1,4}.

Data on Iopamidol and analogues showed that the syn and anti conformers
are populated in similar amounts and that the endo/exo equilibrium exists only
when the anilidic moiety is a tertiary amide\cite{18}. No reference was made to
SYN/ANTI equilibria. There are few characterization of the physical-chemical
properties of NICMs correlated to the molecular conformation in aqueous
solution. Association equilibria as a function of concentration were studied by
osmolality, calorimetry and densitometry, in order to correlate free energy,
enthalpy and volume properties of NICMs solutions\cite{2,4,12,19}.

This study help to understand the role of atropisomerism in the stability of
the concentrated aqueous solutions of NICMs. In particular, we were able
quantify the main species found in concentrated solutions of Iomeprol,
Iopamidol, Iopromide by \textsuperscript{13}C-NMR (including the SYN/ANTI equilibria). The
difficult task of assigning peaks deriving from different conformers was made
possible by the use of simplified precursors. We show that only few of the many
possible forms are present in solution. Furthermore, our data were able to give
information on the nature of the soluble aggregates formed at the concentration
used in commercial preparations, suggesting remarkable similarities with the
structure found by x-ray in the crystallized solutions\cite{20}.
1.7 WORKPLANE

The assessment of the current knowledges of NICMs in the literature shows an abundance of clinical-diagnostic data but a certain lack of physico-chemical data of the properties of these compounds. The success of NICMs in diagnosis is due to the lucky combination of these properties. To clarify the roles of the structural moieties during kinetics processes in solution, to study the molecular details and the differences in behavior is useful.

A collection of thermodynamic data, on various properties of NICMs, allows a direct comparison of physico-chemical properties. So, a methodology of theoretical and experimental study would be possible to develop. Although, as mentioned earlier, all the molecular structures of NICMs are very similar and targeted structural changes may positively modify the self-assembling mechanism and the formation of soluble supramolecular structures relatively stable in aqueous solution. Calorimetric studies allow to classify the NICMs according to their solvophobic degree.

Non-invasive spectroscopic measurements are able to detect transformation kinetic processes and to carry out information on the structural relaxation process obtained from the inelastically scattered light (Brillouin peaks) as a function of both concentration and temperature.

The study conducted on NICM solutions has been addressed to re-evaluate the classical thermodynamic knowledges that can allow to understand the physico-chemical behavior through modern approaches of thermodynamics and molecular dynamics.

To maintain a low toxicity of NICM solutions and then osmolality, self-assembling, which leads to the formation of nano-soluble supramolecular structures, is necessary because the effect is a reduction in the number of particles in solution. The condition is that the packing of the molecules must be not enough effective to transform the soluble nanostructures in crystallization nuclei. The supramolecular nanostructure must be governed by a “quasi chemical” balance during association process.
The intermolecular interactions, NICM-H2O and NICM-NICM, are responsible for the equilibria that occur in solution. In literature there are not enough data in a position to give a comprehensive description of the types of intermolecular interactions. The knowledge about the nature of the interactions would allow to control the self-assembling process. In these terms the molecular regions that are more involved in interactions must be understood by studying changes in function of both temperature and concentration. Moreover, the attention was focused on the atropisomerism (of Iopamidol, Iomeprol and Iopromide) that, in this case, involves carbonyl and amine groups which can form hydrogen bonds.

The pharmaceutical formulation of NICMs consist of a solution characterized by a balance between the different conformational atropisomers, each of which is characterized by an own geometry of interaction. The high concentration of the solutions and the coexistence of atropisomers generate a variety of intermolecular interactions within the system.

The identification of an analytical techniques sensitive to changes in the conformational equilibrium and therefore able to discriminate between different molecular conformation was found, such as the Nuclear Magnetic Resonance (NMR).

Studing also the solid state as a function of temperature and structure types allows to carry out more information about intermolecular interactions. The temperature dependence study on the solid state could show eventual solid-solid transition that could depend from atropisomerism. Furthermore, the identification of the atropisomers involved in the crystallization can be found by x-Ray difractometry examinations.

An induced crystallization (made for Iopamidol; pentahydrated) can help to understand which type of atropisomer in solution generates the effective stacking to form the precipitate.

A conformational order is necessary to get a nucleation center and thus, if the molecular order is maintained during the growth of the nucleation center, the crystalisation process can start. Only few types of atropisomers are probably involved into the crystal to allow the molecular stacking. The knowledge of
atropisomerism of these systems could help to understand its influence on the stability of the concentrated solutions during the shelf-life. The characterization of the conformational kinetics of NICMs could allow to find a physical treatments to ensure maximum effectiveness of these drugs help prevent precipitation.

The roles of the intermolecular interactions would be more clear if an overview of physico-chemical properties was completed.

Comparing thermodynamic and spectroscopic data, curried out in this work, with those in literature and with the molecular dynamics trajectories obtained by prof. Brady (Cornell University, New York), we tried to provide elements for understanding the structure and dynamics of aqueous solutions of NICMs. The overview of physico-chemical properties was expanded.
1.8. BIBLIOGRAPHY


2. MATERIALS AND METHODS

2.1 IOPAMIDOL, IOMEPROL AND IOPROMIDE

The Non-ionic Iodinated Contrast Media (NICM) studied in this work, Iopamidol, Iomeprol and Iopromide, are supplied directly by the producer and were used without further purification. The Iopamidol and Iomeprol aqueous solutions were provided by Bracco Imaging (Milan) and are sold respectively under the names "Iopamiro 370" and "Iomeron 400". In addition to the solutions, that are provided in sterile vials of 10 mL, Iopamidol and Iomeprol powder were also available. Products are also identified by a number, that refers to the concentration of the solution: mg iodine / mL of solution Concentrations were converted and expressed in molality and molarity (moles of NICM / kg water and moles of NICM / L of solution, respectively).

The Iopamidol concentration in the vial as received is 1.48 m which corresponds to a concentration 0.97 M. One gram of Iopamidol solution contains 0.535 g of Iopamidol and 0.465 g of water. The Iomeprol concentration in the vial as received is 1.67 m which correspond to a concentration 1.05 M. One gram of Iomeprol solution contains 0.564 g of Iomeprol and 0.435 g of water. The Iopromide is a product marketed by Schering AG under the name of “Ultravist 370”. This is a solution for infusion and is available in bottles of 200 mL. The molality calculated for this solution is 1.52 and one gram of this solution contains 0.546 g of Iopromide and 0.454 g of water. All dilutions were made from these solutions of NICM by using deionized water.
2.2 NUCLEAR MAGNETIC RESONANCE (NMR)

2.2.1 Instrumentation

All NMR measurements were performed at 300 K on a Bruker Avance III Ultra Shield Plus 600 MHz spectrometer provided with a two channel BBI probe. The assignment was performed by consulting the chemical shift data carried out in DMSO solvent\[1\] and these data used as reference.

2.2.2 Sample preparation

The diluted NICM solutions were prepared by diluting of the vial solution (as received) by using deionized water. The concentrations analyzed were \(8\times10^{-3}\); \(2\times10^{-2}\); \(4\times10^{-2}\); \(6\times10^{-2}\); \(8\times10^{-2}\); \(0.2\); \(0.4\); \(0.6\); \(0.8\) and \(1\) M.

2.2.3 Spectra

\(^1\text{H}\)-NMR 1D-spectra were performed in 10\% D\(_2\)O adding the external reference TSP 1:100. The number of points was 16384, window of 12 ppm centred at 4.7 ppm (water signal). The sample volume was about 600\,\mu\text{L}.

\(^{13}\text{C}\)-NMR 1D-spectra were performed in 10\% D\(_2\)O adding the external reference TSP 1:100. The spectra were recorded by proton decoupling. The number of points was 65536, window of 200 ppm with transmitter at 100 ppm. The sample volume was about 600\,\mu\text{L}.

The temperature dependence study was performed in a temperature range from 278 K to 338 K by 1D \(^1\text{H}\)-NMR and 1D \(^{13}\text{C}\)-NMR experiments. Internal reference was TSP 1:100. The sample volume was about 600\,\mu\text{L} with 10\% of D\(_2\)O.

The concentration dependence study was performed in a concentration range from 0.008 M to 1 M by 1D \(^1\text{H}\)-NMR and 1D \(^{13}\text{C}\)-NMR experiments. Internal reference was TSP 1:100. The sample volume was about 600\,\mu\text{L} with 10\% of D\(_2\)O.

Longitudinal and transversal relaxation rates (both \(^1\text{H}\) and \(^{13}\text{C}\)) were measured by inversion recovery and CPMG pulse sequence as function of
Materials and Methods

Concentration from 8 mM to 1 M. $^1$H decoupling during relaxation delay was avoided during $^{13}$C measurements.

NOESY (Nuclear Overhauser Effect) experiment were performed for a Iopamidol–Iomeprol mixture (1:1 molar ratio), in 10% D$_2$O adding the external reference TSP 1:100. The number of points was 1024, window of 12 ppm centred at 4.7 ppm (water signal). Mixing time was $= 100-300$ ms.

HOESY (Heteronuclear Overhauser Effect) experiment were performed in 10% D$_2$O adding the external reference TSP 1:100. The number of points was 1024, window of 12 ppm centred at 4.7 ppm (water signal).

Diffusion coefficients were carried out by DOSY (Diffusional Ordered Spectroscopy) experiment [2][3]. All experiments were performed by using concentrations range from 0.08 M to 1M Of NICM solutions in H$_2$O (10% D2O). TSP was added in ratio 1:100 as internal chemical shift reference.

The intensity of the signals was plotted as a function of the gradient strength $G$ and regression analysis was performed using Topspin (Bruker) with the equation [3] 2.1:

$$I = I_0 \exp[-Dq^2(\Delta - \delta/3 - \tau/2)]$$

$I$ and $I_0$ are the intensities of the signal for each gradient strength used in the experiment and for 2% gradient strength, $\Delta$ and $\delta$ are the big and the little delta of the STEbp [4] experiment (STimulated Echo with bipolar gradients), $\tau$ is the gradient pulse separation and $q = 2\pi\gamma G \delta$. The STE version of the sequence was used to avoid thermal motions in solution due to the low viscosity of TSP.

The viscosity of the solution was determined by the diffusion coefficients $D$ of the internal TSP assuming a hydrodynamic radius of 0.351 nm [5] using the equation 2.2:

$$D = \frac{k_B T}{f_T} \quad f_T = 6\pi\eta r_H$$
where $k_B$ is the Boltzmann factor, $T$ the absolute temperature and $\eta$ is the viscosity of the medium and $r_H$ is the hydrodynamic radius of the molecule, here assumed of spherical shape\textsuperscript{6}.

In order to roughly estimate the molecular weight of the species observed the apparent molecular weight $M$ was calculated\textsuperscript{7} \textsuperscript{8} as:

$$M = \left( \frac{k_B T}{6 \eta \pi F D} \right)^{3/2} \frac{4 \pi N_A}{3 (\nu_2 + \delta_1 \nu_1)}$$

where $N_A$ is Avogadro’s number, $\nu_2$ and $\nu_1$ are the partial specific volumes of the molecule (solute) and solvent (water) respectively, and $\delta_1$ is the fractional amount of solvent bound to the molecule (solvation number). $F$ is the shape factor, or Perrin factor, which is defined as the ratio of the friction coefficient of the molecule to that of a hard sphere with equivalent mass and partial specific volume. In this study NICM molecules were approximated to a sphere and $F=1$ was used.

Partial specific volumes $\nu_2$ and $\nu_1$ of the material and the solvent were assumed $1 \times 10^{-6}$ m$^3$g$^{-1}$ and $0.36 \times 10^{-6}$ m$^3$g$^{-1}$, respectively\textsuperscript{9} and a value of 0.15 g water/g substance was used for the solvation number\textsuperscript{7}.

\textbf{2.3 MOLECULAR DYNAMICS}

The molecular dynamics simulations\textsuperscript{10} \textsuperscript{11} were performed in collaboration with Professor J. W. Brady’s laboratory at Cornell University (Ithaca, New York, USA)

\textbf{2.3.1 programs and simulations}

The molecular dynamics simulations were performed by using the molecular dynamics and mechanics CHARMM program (Chemistry at Harvard Molecular Mechanics) and potential energy has been modeled by using the
CHARMM22 force field type. The TIP3P model was used to simulate the water molecules.

### 2.3.2 Theory

The computer simulations of molecular dynamics allow us to study the evolution of a system over time (fluctuations, diffusion and velocity). Furthermore, it allows us to predict the static and dynamic properties of substances as interactions between molecules. The behavior of the molecules as a function of time is well described by the quantum-mechanical Schrödinger equation which is time-dependent. However, it is extremely difficult to solve for large molecules, so the atomic motions described by the classical laws of mechanics were assumed.

The theoretical basis that relates the physical properties of the system to both trajectories and velocity of the atoms is based on the ergodic theorem (sampled on a sufficiently long period of time). According to this theorem, given a long enough period of time, a system comes arbitrarily to populate each point of the phase space. The time spent in each region of phase space is governed by the Boltzmann distribution. In this way, all the physical observables can be calculated by taking the appropriate average time on the atom trajectories of a system.

### 2.3.3 Experimental setup

The simulations were carried out on the aqueous solutions of Iopamidol at various concentrations: 0, 1 and 0.5 molality. The molecules of Iopamidol (1, 15 and 30) were randomly oriented and placed in a cubic box with 1273, 1666 and 1671 water molecules respectively (Table 2.1).

**Table 2.1:** molecular dynamic simulations of Iopamidol.

<table>
<thead>
<tr>
<th>Concentration (m)</th>
<th>( N_{\text{iopamidol}} )</th>
<th>( N_{\text{water}} )</th>
<th>Box size (Å)</th>
<th>Density (g/cm(^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1</td>
<td>1273</td>
<td>34.01</td>
<td>1.000</td>
</tr>
<tr>
<td>0.5</td>
<td>15</td>
<td>1666</td>
<td>39.07</td>
<td>1.160</td>
</tr>
<tr>
<td>1.0</td>
<td>30</td>
<td>1671</td>
<td>40.92</td>
<td>1.295</td>
</tr>
</tbody>
</table>
To avoid the overlap among molecules, the minimum distance between Iopamidol-Iopamidol, Iopamidol-water and water-water have been regarded. With this procedure the molality of these systems is: 0.044 m, 0.5 m, 0.997 m. The simulations were performed for 4 ns at 300 K and the configurations were saved every 0.2 ps.

2.4 FTIR-ATR (ATTENUATED TOTAL REFLECTANCE)

The IR measures were carried out in collaboration with Dr. Marco Paolantoni at the Department of Chemistry, University of Perugia.

2.4.1 Instrumentation

Infrared spectra were recorded with a Bruker IFS28 equipped with a DTGS detector; each spectrum was the average of 50 scans recorded with 2 cm\(^{-1}\) resolution. A multi-reflection Specac Gateway\(^{TM}\) ATR system with a ZnSe crystal was employed.

2.4.2 Sample preparation

The samples tested were Iopamidol solutions as received. The diluted solution were prepared from the original formulation by using deionized water. The concentration was 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 M.

The Iopamidol concentrated solution was used in the experiments at the temperatures of 25, 35, 42, 55 and 67 °C.

The analysis of the spectra was only qualitative to find dependences from concentration and temperature.

2.4.3 Theory

IR spectra were recorded in order to study the temperature and concentration dependence of both C=O and NH groups. Information on the involvement of these two moieties in intermolecular interactions can be obtained
monitoring the red and blue shift of the band signals of NICMs with temperature and concentration. The interaction, for example a hydrogen bond involving both carbonyl and amine groups, causes a change in the vibrational modes of the C=O, CO-CNH and N-H moieties. The consequences are changes in the spectra. A blue shift of a band (with concentration or temperature), means that its bond strength is becoming more stronger due to the destruction of a secondary bond and vice versa. The secondary bonds are influenced by temperature and concentration and this fact is exploited to probe kinetics processes.

2.5 **BRILOUIN SPECTROSCOPY**

Measurements were performed in the light scattering laboratory of GHOST group\[12\] at the Department of Physics, University of Perugia.

2.5.1 **Instrumentation**

Polarized diffusion spectra (I_{VV}) at low frequencies (0.3-60 GHz) were recorded by analyzing the scattered light by using a Fabrt-Perot interferometer. The spectra were recorded by diffusion geometry of 180°. In the figure 2.1 a scheme of the optical bench used is shown.
The light source used in this work consists of solid state laser with 200 mW of power on the single longitudinal mode and 532 nm of wave length. The light beam comes to a beam-splitter and it is splitted into two parts. One beam is direct to the sample, the other is direct to the interferometer as beam reference and is used also to align the mirrors.

A lens is used to focus the beam on the sample and to collect, by back scattering, the scattered light which, through a mirror group, comes to the entrance pinhole of interferometer.

The polarization of incident and scattered beam were selected by a polarizer and an analyzer respectively. The frequency analysis of the scattered light of the sample was carried out by Sandercock tandem interferometer triple step model (Fabry-Perot) that consists of a pair of interferometers Fabry-Perot model. Each interferometer is composed by two parallel mirrors at \( L \) distance that can be varied.

2.5.2 Theory

The light scattering techniques are based on the interaction between the oscillating electric vector of incident electromagnetic radiation with the electrons of the sample. This interaction generates an oscillating dipole moment in phase
with the electric vector and the point of interaction becomes a source of radiation scattered in all the directions.

In Brillouin spectroscopy the light emitted by the source, most commonly a laser, passes through the polarizer to select the polarisation required for the experiment. The polarisation vector of the light source can be oriented parallel (H) or perpendicular (V) to the scattering plane. If the incident light is polarised V and the diffused light is H, the experiment is known as $I_{VH}$ or depolarised scattering. When both polarisations are V the experiment is called $I_{VV}$ or polarised scattering.

The characteristics of the scattered light depend upon the time scales of the movement of molecules, thus by means of time correlation functions of the scattered field or intensity a description of such dynamical aspects is achieved. The results correspond to measurements in backscattering configuration in the visible range. $I_{VV}$ was acquired for NICM solutions as received (Iopamidol and Iomeprol). The elastic scattering measurements provide information on molecular mass, shape and size of the scattering molecules, while the inelastic diffusion method describes the diffusion of system and its structural relaxations. An example of Brillouin spectra is shown in Figure 2.2.

![Figure 2.2: Brillouin spectra of a trehalose solution at temperature increasing (from 0 to 1).](image-url)
The Brillouin spectra in figure 2.2 show intensity vs frequency trend as a function of temperature. The central peak is the elastic contribution while the two near symmetrical peaks are the inelastic contributions.

The changes of the frequency and the width of the Brillouin peaks are due to the temperature. When temperature increases both frequency and width of Brillouin peaks decrease. These changes contain information of the kinetic processes in the system. By plotting of frequency (or width) vs time is useful to reveal a kinetic process at fixed temperature as in the case of this work (Figure 2.3). If a kinetic process is present, changes in terms of frequency and width are detectable.

![Figure 2.3](image.png)

**Figure 2.3:** example of kinetics study, performed in this work, of Iopamidol at 85 °C. (left) Brillouin shift for VIS data of Iopamidol concentrated solution as a function of time, from the DHO model. (right) HWMH for VIS data of Iopamidol concentrated solution as a function of time, from the DHO model. by collaboration with University of Perugia (Prof. D. Fioretto).

### 2.5.2 Sample preparation

2-3 mL of Iopamidol or Iomeprol solution were added into a test tube which was hermetically sealed by melting the glass at half level of test tube length
size. Hermetic seal was necessary because the experiments were performed over long time at high temperatures.

Measurements were performed between 25 °C and 85 °C by using a thermocryostat and a copper sample holder. Each sample was thermalized for 15 minutes before the measurement.

2.6 X-RAY DIFRACTOMETRY

Measurements of x-Ray diffraction were carried out in collaboration with the Laboratory of Biocrystallography, at Department of Chemistry, University of Trieste and with XRD1 beamline of Elettra S.p.A. Trieste (Synchrotron Radiation). Additional experiments of real time temperature transition on powder crystalline forms were carried out by using the SWAXS beamline.

2.6.1 Materials and methods

Crystals of Iomeprol were obtained from a water solution of the molecule, concentration of 100 mg/mL, using the sitting drop method. A drop of 7 µL was set to equilibrate against a 1mL reservoir solution containing 90% ethanol – 10% water (v/v). Crystals of the compound were formed after 20 days. The crystal structure of Iomeprol was first determined at room temperature using a molybdenum source (\(\lambda = 0.71073\) Å) at the Laboratory of Biocrystallography, University of Trieste. Subsequently, another dataset of a similar crystal was collected at 100 K using a synchrotron light source (XRD1 beamline at Elettra, Trieste), at a wavelength of 0.8000 Å. For the room temperature data collection, a crystal was mounted on a glass fibre with acrylic glue. When frozen at 100 K by a nitrogen stream, the crystal was protected by addition of Paratone and collected with a nylon loop before flash freezing. Diffraction data were indexed and integrated using DENZO\textsuperscript{13} and scaled with SCALEPACK\textsuperscript{14}. The structure was solved by direct methods using SHELXS\textsuperscript{14}. The structure was refined by full-matrix least-squares methods on \(F^2\), using SHELX-97\textsuperscript{15}. All non-hydrogen atoms were treated anisotropically. In the final refinement, hydrogen atoms were
included at calculated positions, with the torsion angle of the methyl groups free to refine. The software XABS was used to apply an empirical correction to the experimental intensities, in order to correct data for X-ray absorption.

2.7 SOLUTION CALORIMETRY

2.7.1 Isothermal calorimetry: Instrumentation

The calorimetric measurements were performed by the microcalorimeter batch LKB 10700-2. It is a mixing calorimeter, almost isothermal, built by Ingemar Wads of Lund University. This calorimeter allows to make very precise measurements. Although this calorimeter dates back to the seventies, is still one of the most sensitive calorimeters built and marketed. It is an instrument designed to measure the heat released or absorbed during an endothermic or exothermic process using about 1-2 mL of solution.

In this work, the calorimeter was used to determine the heat of dilution involved between NICMs and water.

The instrument consists of the following parts:

• thermostatic apparatus containing the calorimetric unit
• control unit
• microamperometer Keithley 150B
• thermostat Haake F3
• data interface Picolog
• PC to collect and to monitor the measures

The calorimetric unit is inserted in the thermostatic system that consists of an air-chamber with high thermal capacity whose temperature is controlled by a temperature sensor and kept constant by a circulating air flow. The calorimeter was used at 25 °C and 37 °C.

The microcalorimetry unit consists of a horizontal cylinder with a in which there is an aluminum block insulated by a layer of polystyrene foam. Two cells
are placed inside the aluminum block: one is the reference and the other is to measure. In relation to the geometry of the cylinder, the cells are arranged symmetrically and are not visible.

The cells are identical and made in gold to have maximum both thermal conductivity and chemical inertness. They are composed by two compartments with different volume. The capacity of the largest compartment is 4 mL, while the capacity of the smallest one is 2 mL. The reference cell is usually filled with the solvent used for preparation of the solutions, in this case water, and its function is to minimize the thermal imbalance of the system during mixing. A difference of temperature between the two cells can be generated during the mixing mode and it is converted into a potential difference.

### 2.7.2 Isothermal calorimetry: Theory

The heat of dilution ($\Delta H_{\text{dil}}$) data were elaborated in terms of apparent molar enthalpies which allow to obtain the partial molar quantities. The partial molar quantities were calculated by following method.

Enthalpy is a thermodynamic property whose change can be defined as the heat exchanged with the environment at constant pressure of a system. The system is free to change its volume during the process.

There is a simple equation that relates enthalpy and internal energy:

$$ H = U + pV $$  \hspace{1cm} 2.4

where $U$ is internal energy of the system, $p$ is the pressure and $V$ the volume and are all state functions. This fact implies also enthalpy is a state function.

The partial molar enthalpy\textsuperscript{[16]} concept is introduced when mixtures characterized by two or more components are studied. In these terms, partial molar enthalpy can be defined as contribution of a mixture component $\overline{H}_i$:

$$ \overline{H}_i = \left( \frac{\partial H}{\partial n_i} \right)_{T,p,n} $$  \hspace{1cm} 2.5
\[ L_i = H_i^0 - H_i \]

2.6

The change is measured by measuring heat absorbed or evolved during a dilution, the enthalpy. Relative partial molar enthalpy of the process is carried out.

During a mixing process, that involves one solute and one solvent, the enthalpy difference is:

\[ \Delta H_{mix} = H_{final} - H_{initial} = n_1 H_1 + n_2 H_2 - n_1 H_1 - n_2 H_2 \]

2.7

where \( H_1 \) is molar enthalpy of the pure solvent and \( H_2 \) that of the pure solute.

For pure solvent \( H_i^0 \) value is equal to \( H_1 \). Thus:

\[ \Delta H_{mix} = n_1 L_1 + n_2 \left( H_2 - H_2^0 \right) \]

2.8

adding \( H_2^0 - H_2 \) term to the equation 2.8:

\[ \Delta H_{dil} = n_1 L_1 + n_2 \left( H_2 - H_2^0 + H_2^0 - H_2^0 \right) = n_1 L_1 + n_2 L_2 - n_2 L_2 \]

2.9

The partial derivative, in respect to \( n_1 \) with constant \( n_2 \) value, of the equation 2.9 is:

\[ \left( \frac{\partial H}{\partial n_1} \right)_{n_2} = L_1 \]

2.10

by maintaing constant the \( n_1 \) value and deriving the equation 2.9 in respect to \( n_2 \), the expression becomes:
\[
\left( \frac{\partial H}{\partial n_2} \right)_{n_1} = \bar{L}_2 - L_2 \quad 2.11
\]

to obtain \( \bar{L}_2 \) by elaborating experimental data, relative molar enthalpy of the pure solute must be known.

Assuming \( \lim_{n_2 \to 0} L_2 = 0 \):

\[
\lim_{n_2 \to 0} \left( \frac{\partial H}{\partial n_2} \right)_{n_1} = -L_2 \quad 2.12
\]

\( \bar{L}_2 \) can be calculated by both plotting \( \Delta H \) vs \( n_2 \) and calculating the slope of the curve at fixed \( n_2 \) value.

The calorimetric data are often analyzed in terms of apparent molar enthalpy which is defined as:

\[
H_\Phi = \frac{H - n_1 H_1}{n_2} \quad 2.13
\]

Rearranging the equation 2.13:

\[
n_2 H_\Phi = H - n_1 H_1 = n_1 \bar{H}_1 + n_2 \bar{H}_2 - n_1 H_1 \quad 2.14
\]

For \( \lim_{n_2 \to 0} H_\Phi = H_\Phi^0 = \bar{H}_2^0 \) it is possible to write:

\[
n_2 H_\Phi - n_2 H_\Phi^0 = n_1 \bar{H}_1 + n_2 \bar{H}_2 - n_1 H_1 - n_2 \bar{H}_2^0 = n_1 \bar{L}_1 + n_2 \bar{L}_2 \quad 2.15
\]

The experimental heat of dilution data are analyzed in terms of relative apparent molar enthalpy, defined by the follow expression:

\[
L_\Phi = H_\Phi - H_\Phi^0 \quad 2.16
\]

\[
n_2 L_\Phi = n_1 \bar{L}_1 + n_2 \bar{L}_2 = L \quad 2.17
\]

\[
L_\Phi = \frac{L - L^0}{n_2} = \frac{L}{n_2} \quad 2.18
\]
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\[ \bar{L}_2 = \left( \frac{\partial L}{\partial n_2} \right)_{n_1} = \frac{\partial}{\partial n_2} (n_2 L_\phi) = L_\phi + n_2 \left( \frac{\partial L_\phi}{\partial n_2} \right)_{n_1} \]  

2.19

by inserting the expression 2.15 the equation becomes:

\[ n_1 \bar{L}_1 = n_2 L_\phi - n_2 \bar{L}_2 = n_2 L_\phi - n_2 \left[ L_\phi + n_2 \left( \frac{\partial L_\phi}{\partial n_2} \right)_{n_1} \right] \]  

2.20

\[ \bar{L}_1 = -\frac{n_2}{n_1} \left( \frac{\partial L_\phi}{\partial n_2} \right)_{n_1} \]  

2.21

Relative partial molar enthalpy is related to the apparent molar enthalpy by the following relation:

\[ \bar{L}_2 = \left( \frac{\partial L_2}{\partial m} \right)_{n_1} = L_\phi + m \left( \frac{\partial L_\phi}{\partial m} \right)_{n_1} \]  

2.22

\( \bar{L}_2 \) and \( L_\phi \) can be carried out by experimental heat of dilution measures during a mixing process that involves a solution with an initial concentration \( m_i \) and the pure solvent. Their mixing lead to a solution with a final concentration \( m_f \).

\[ \Delta H_{\text{dil}} = H_{\text{finale}} - H_{\text{iniziale}} = L_\phi (m) - L_\phi (m_i) \]  

2.23

To carry out \( L_\phi \) value a series of dilution are neccessary until the solution will be extremely dilute to applicate the \( \lim_{m \to 0} (m) = 0 \) relation:

\[ \lim_{m \to 0} [(\Delta H_{\text{dil}})] = \lim_{m \to 0} [(L_\phi (m) - L_\phi (m_i)] = -L_\phi (m_i) \]  

2.24

The \( \Delta H_{\text{dil}} \) values related to each dilution process are summed and the vale at \( m=0 \) is extrapolated. This value is the zero in the graph of \( L_\phi \) (kJ mol\(^{-1}\)) as function of concentration (molality, mol\(_{\text{solute}}/\text{kg}_{\text{solvent}}\)).

2.7.3 Isothermal calorimetry: Methods

The control unit includes another unit to rotate the cylindrical block. An electric motor, that allows the cylinder to rotate, is driven by a command: a clockwise rotation of 400 °, a counterclockwise of 440 ° and then again a
clockwise rotation of 40°. For viscous solutions may be necessary to repeat several times the rotation mode to optimize the mixing process and achieve the proper thermal equilibrium. The instantaneous amount of heat that develops during the mixing process is given by:

$$\frac{dQ(t)}{dt} = K \cdot \Delta T(t)$$  \hspace{1cm} 2.25

where $Q(t)$ is the amount of heat involved in the dilution process, $K$ is the heat transfer constant, $\Delta T(t)$ is the difference of temperature that is proportional to the change in electrical potential by the relation:

$$\Delta T = \int_{t_1}^{t_2} E dt$$  \hspace{1cm} 2.26

where $E$ is the electric potential, $t_1$ and $t_2$ are the initial and the final time of the reaction.

The variation of potential difference is represented by area $A$ that is subtended at a calorimetric curve from $t_1$ to $t_2$. The heat involved in the chemical reaction is directly proportional to $A$:

$$Q = \varepsilon A$$  \hspace{1cm} 2.27

$\varepsilon$ is the constant calibration. The constant $\varepsilon$ is determined by a series of calibrations that are carried out by measuring the amount of heat developed by passing an electric current through a resistance.
Both the intensity and the lifetime of the electric current are known.
The amount of heat, that develops during the calibration phase, is determined by the equation that relates the heat with the intensity of electric current:

$$Q_{cal} = RI^2t = \varepsilon A_{cal}$$  \hspace{1cm} 2.28

$I$ is electric current intensity known, $R$ is the resistance value and $t$ is the time set for the calibration.
The average value of the constants $\varepsilon$, calculated from each calibration, is determined under the same conditions of both temperature and amplification scale adopted in the experimental measurement. The experimental result is obtained by integrating the area subtended at the curve of each calorimetric experiment whose signal is a function of the time.

This calorimeter works at high sensitivity conditions and frictional heat must be considered. The difference between the conditions of the reaction cell and the reference cell, due to the frictional heat, cannot be neglected. The frictional heat is due to the viscosity and density of the solution. The frictional heat can be measured by subtracting the peak of Africa, due to the area on the additional mixing, from the area of the curve measuring:

$$ A = A_{\text{dil}} - A_{\text{fric}} $$

In this way the area $A$ is only due to the heat generated during the reaction. The reaction heat value is calculated by relating the amount of the heat developed during calibration with the amount of heat flowing after dilution:

$$ Q_{\text{dil}} = \frac{Q_{\text{cal}} \cdot A_{\text{dil}}}{A_{\text{cal}}} $$

The heat of reaction is normalized to the number of moles of the species involved in the mixing process and the ratio correspond to the enthalpy of dilution process:

$$ \Delta H_{\text{dil}} = \frac{Q_{\text{dil}}}{n} $$

The experiments were done at 25 °C and 37 °C that were the two internal temperatures of the calorimeter adopted. Starting from commercial solution of NICMs in the vial, or from a solution prepared in the laboratory by dissolving the powder in water, a series of consecutive dilutions for each NICM was carried out measuring the heat released or absorbed from the system.
2 mL of the initial solution were introduced into the largest compartment of the reaction cell and 1 mL, or 2 mL, of milliQ water were introduced into the lower compartment.

In the reference cell both compartments were filled with a volume of MilliQ water corresponding to the compartments of the reaction cell. To introduce solutions inside the compartments of the two cells an Hamilton microsyringes of 2.5 mL was used. The microsyringes was weighed before and after injection in the calorimeter to know exactly the weight of the solution, or milliQ water, introduced in the cells. The filled cells are closed with rubber stoppers. To ensure a better reproducibility in the mixing process the position of both solution and water remained the same in each cell. Once the cells are loaded, the cylinder block is closed and the system is equilibrated for about 15 hours.

The signal, in $\mu V$, versus time is analyzed to check the thermalization. The thermalization is reached when the signal is constant with the time. A calibration can be done by using an electric current that develops an amount of heat similar to that which presumably could be developed during the dilution process of the analyzed system. Another waiting time is necessary to allow the system to reach thermal equilibrium again. An appropriate scale is chosen and the measure can be done. The pattern of the calorimetric signal with the time is analyzed on the PC monitor.

An initial mixing process is done by generating the first peak in the signal vs time graph. A second mixing process is done at half the height of the first peak. Other successive rotations, of the cylindrical block, are performed at intervals of 2 minutes until the signal does not show significant thermal effect. The amount of frictional heat is considered when the background scale is 30 $\mu V$. It is measured after both 15-20 minutes and 30-40 minutes from the end of the sample measure and.

The volume of solution, required for the next dilution, is taken from the cell reaction at the end of the measure. The two cells are washed using a syringe by three milliQ water washes and one by acetone. The cells are dried by filtered air flow through the holes of the respective compartments.
The frequency of signals recording was one data per second. The data were acquired by a computer and saved in "*.dat" format. The analysis of calorimetric data was performed by using the program Origin 6.0 (Microcal Software, Inc).

### 2.7.3 Differential Scanning Calorimetry (DSC): Instrumentation

Measurements of the specific heat of the NICM solutions were performed by the heat flux calorimeter “micro-DSC III” manufactured by Setaram.

This calorimeter can measure some thermodynamic quantities (enthalpy, specific heat ..) as a function of a controlled change of temperature. Both sample and the reference are subjected simultaneously to a temperature program. The signals are related to the difference in thermal response of both sample and reference. The calorimetric block is a metal chamber characterized by an high heat capacity. Into the metal chamber two cylindrical steel cells are placed. The capacity of the cells is 1 mL that usually are filled with 0.7-0.8 g of solution. The cells used for measurements are provided by both a screw cap and a rubber gasket that make the cells resistant to a pressure of 20 bar. The cell temperature is controlled by many thermocouples in series and varies according to the set schedule. The temperature of the cells is maintained equal to the calorimetric block by using the heat flux transducers. The temperature scan is performed at a constant speed. During the experiments the temperature difference, between the sample and the reference, is measured and corresponds to a heat flow that depends on their thermal capacity. The signal originated from the temperature difference is amplified and processed by a computer that provides data of heat flow, outgoing or incoming, from the sample compared to the reference.

During the experiments the sample can undergo a transition which correspond to a change in thermal capacity. During a transition the heat flow changes and the process can be endothermic or exothermic and described by the appearance of a curve in the output signal.

The calorimetric system is connected to an analogical-digital interface Setaram CS32 type that allows the amplifier of the instrument to send output signals in parallel to both the recorder and the computer. The computer allows to
program the temperature, to carry out complex long-term cycles and to do data recording.

### 2.7.4 DSC: Theory

The *Heat capacity* is a function which relates an amount of heat (evolved or absorbed) with a temperature variation at constant pressure. The heat absorbed by a system is proportional to $\Delta T$:

$$Q = \int_{t_i}^{t_f} C_p \,dT = C_p \,(T_2 - T_1)$$  \hspace{0.5cm} 2.32

The relative heat, $Q$, is carried out by integrating the thermograms areas. $Q$ is defined as heat capacity difference between reference and sample cell times $\Delta T$, at average temperature $T_{\text{init}} + \frac{\Delta T}{2}$:

$$Q = \Delta C_p \cdot \Delta T$$  \hspace{0.5cm} 2.33

Heat capacity is proportional to the sample mass subjected to temperature change ($\Delta T$).

$$\Delta C_p = m \cdot \Delta c_p$$  \hspace{0.5cm} 2.34

$\Delta c_p$ is defined as the difference between sample and reference (water in this case) specific heats. In these terms, specific heat can be defined as the amount of heat necessary to increase the temperature of 1 K of a experimental unit mass. By normalizing the experimental $Q$ to the sample mass in the cell and by heat capacity difference, specific heat difference between sample and reference can be carried out. The condition is to have the same mass for both sample and reference within the experimental limits

$$\Delta c_p = \frac{\Delta C_p}{m}$$  \hspace{0.5cm} 2.35

that can be written as:
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\[ c_{p, \text{soluzione}} = c_{p, \text{acqua}} - \frac{\Delta C_p}{m} \]  \hspace{1cm} 2.36

where \( m = m_{\text{campiono}} = m_{\text{riferimento}} \) and for \( c_p \) water values literature data are used.

By apparent specific heat definition the following equation was carried out:

\[ \phi c_p = \frac{c_{\text{pot.}} - m_{\text{H}_2\text{O}} \cdot c_{\text{H}_2\text{O}}}{m_{\text{MDC}}} \]  \hspace{1cm} 2.37

where \( m_{\text{H}_2\text{O}} \) is the water mass and \( m_{\text{NICM}} \) is the NICM mass into the solution at a given concentration. \( \phi c_p \) is an apparent property because expresses the excess heat relative to water mass contained in the solution. As for all apparent quantities, the water contribution to the heat capacity of the sample is not influenced by the presence of the solute.

The changes of \( \phi c_{p, \text{NICM}} \) with concentration allow to carry out \( c_p \):

\[ c_{p, \text{solution}} = m_{\text{NICM}} \cdot \phi c_{p, \text{NICM}} + m_{\text{water}} \cdot c_{p, \text{water}} \]  \hspace{1cm} 2.38

The experimental data elaboration is performed by integrating the heat flux peaks which correspond to the temperature steps. The integration needs a fitting of baseline especially for very diluted solutions. By a polynomial function the selected experimental points of each isothermal process can be approximated (figure 2.5)
By integrating after correcting the base line the heat associated to each temperature step ($\Delta T = 5^\circ C$) can be carried out. Apparent specific heat can be calculated.

By knowing the heat capacity of the water contained in the reference cell, the heat capacity of the sample can be carried out. By knowing the heat capacity of the solution, the heat capacity of the water must be subtracted to carry out the specific heat of the NICM analyzed at fixed temperature and concentration.

2.7.5 DSC: Experimental method

The accuracy of the calorimeter was determined by preliminary thermal cycles using only deionized water in both cells obtaining an initial thermogram. An amount of water (50 µL) is removed from the sample cell, a new thermogram is recorded and a comparison is made between the initial and the final thermograms as shown in figure 2.6.
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The experimental results are compared to the specific heat of the water ($c_p = 4.18 \text{ J g}^{-1} \text{ K}^{-1}$) that is reported in literature to verify the accuracy of the calorimeter.

If the test result confirms an high degree of accuracy, the results of measurements are considered valid and is not necessary to apply a new calibration or correction formulas to the obtained data.

After the test the measurements of specific heat can be performed. The NICM solution is injected into the sample cell while the reference cell is filled with Deionized water (about 0.8 g) to balance both the weight of the cells and the thermal signal.

The difference in weight between the cells is kept within the limit of 1 mg.

Thermal cycles, between 10 °C and 90 °C, are performed by steps of 5 °C (SR = 0.5 °C/min). For each step an isothermal process is maintained for 20 minutes (Figure 2.7).
The Setsoft data acquisition software allows to choose both the frequency of acquisition (data/sec) and to follow in real-time the development of the measurement. The data are grouped into files that are normalized, formatted, transferred and processed by another computer using Origin 6.0 software.

![Figure 2.7: thermal cycles of heating and cooling of a diluted Iopamidol solution with water; the ratio Iopamidol solution:water is 1:1.](image)

The specific heat, which in this case is apparent, provides an accurate determination of the change in enthalpy (and entropy) of the system with temperature. This technique is characterized by an high sensitivity for the detection of changes in energy of the system is pure state or in solution.

Measurements of apparent specific heat were carried out as a function of concentration at four different temperatures (25 °C, 29 °C, 33 °C and 37 °C) to verify the presence of aggregation phenomena.
The thermal cycles of this experiment were carried out on both concentrated and diluted NICM solutions. For concentrated solutions the thermograms were recorded by a single thermal cycle (heating than cooling), while for diluted solutions the thermal cycle was repeated twice to minimize the area errors calculating the average area for each temperature above illustrated. The points on the graph, $C_p$ vs conc., solutions to relatively low concentrations (below the value of 1 m) were calculated by taking the average value in the four temperature ramps performed in each experiment (two in two heating and cooling).

In these experiments the same volume of solution or water, for sample and reference, was used. The difference in weight was considered in the equations to calculate $\phi_{cp}$.

2.8 CALORIMETRY ON SOLID SAMPLES

2.8.1 DSC: Instrumentation

Calorimetric measurements were carried out with a Perkin-Elmer DSC 6. The Pyris software version 3.81 from Perkin-Elmer was used with Windows NT 3.5. The thermal unit was thermalized with an external thermocryostat in with the coolant was kept at 0 °C; a nitrogen flux (20 ml/min) was used as a purge gas for the furnace. Temperature scans were run on samples weighing about 10 mg and sealed in aluminium Perkin-Elmer DSC pans; an empty aluminium pan was used as a reference. Temperature and heat flow calibration were carried out according to standard procedures for DSC 6.

2.8.2 DSC: Experimental method

Isothermal and cooling calorimetric physical aging (at 140 C°) were performed to study the glassy transition of Iopamidol and Iomeprol at various scanning rate. The samples were analyzed for both glass and powder of Iopamidol and Iomeprol.
**Materials and Methods**

**Isothermal Aging of amorphous Iopamidol and Iomeprol at 140 °C:**
20 mg of concentrated solution of NICM were directly treated into the DSC 6 calorimeter at 110 °C for 17 hours to evaporate the water and to obtain the amorphous solid. The heating and cooling rate were set to be constant in the temperature range from 100 °C to 200 °C and was 20 °C/min. The sample was maintained at 140 °C for several times: 30, 60, 90, 120, 120 and 300 minutes. The temperature value of 140 °C was chosen to allow a significant aging of NICMs. If temperature is close to that glassy transition the aging process is enhanced over time. This temperature set up was used for both Iopamidol and Iomeprol.

**Aging by cooling of amorphous Iopamidol and Iomeprol at various temperature scanning rates:** the amorphous samples of NICM are treated at 110 °C for 17 hours. Temperature cooling cycles were set from 200 °C to 80 °C by using different temperature scanning rates (20, 10, 5, 2, 1, 0.5 °C/min) followed by heating from 80 °C to 200 °C with 20 °C/min scanning rate.

**Aging by cooling of Iopamidol and Iomeprol powder at various temperature scanning rates:** the aging experiment, at various temperature scanning rates, were performed also on Iopamidol and Iomeprol powder. The temperature setup of the thermal cycles, as in the amorphous solid cases, were maintained constant at 20 °C/min for heating and 20, 10, 5, 2, 1, 0.5 °C/min for cooling.

**2.9 MICRO-RAMAN SPECTROSCOPY**

Measurements were performed in collaboration with the micro-Raman spectroscopy laboratory at Department of Industrial and Information Engineering, university of Trieste, with Dr. Alois Bonifacio by using a MicroRaman Renishaw InVia. The light source was a Laser High Power NIR 785 nm with power on the sample of 90 mW. The acquisition times were between 10 and 60 seconds.

The simulated spectra were carried out by using Gaussian 03 for Windows software (serial version). Theory level: functional density B3LYP, bases used 6-
Materials and Methods

31+G*. The program calculates the Raman spectrum for a molecule, giving the respective vibrational modes. The calculation was performed by using crystallographic coordinates carried out by structural x-Ray data of the NICM analyzed.

Glasses of Iopamidol and Iomeprol were realized by heat treatment at 110 °C for 17 hours into DSC 6 calorimeter, as for calorimetric cases. Iomeprol crystalline powder was used as received. Iopamidol pentahydrate was prepared by crystallization of a Iopamidol concentrated solution. The pentahydrate crystal was directly analyzed as precipitate without drying.

Additional experiments of real time temperature transition on both single crystal and powder crystalline forms were carried out by using a custom-made temperature controller operated by a PC. Scanning was performed in the range 30-130 °C at scanning rate of 1 °C/min.
2.10 BIBLIOGRAPHY


3. RESULTS AND DISCUSSION

PREMISE

The large variety of methodological approaches used to study the NICMs in this work makes difficult the presentation of the results without a continuous cross-reference of sections and experimental results. Although following an arbitrary order of presentation, the results are presented from the data of the NICMs in dilute solution to the more concentrated systems and to the solid state properties. Therefore, some repetitions in the particular methods used is unavoidable. This chapter of results contains altogether comments and annotations that would have been more correctly allocated in a separate chapter entitled “Discussion”.

The results are presented in the following order. First, the characterization is reported of the Iopamidol, Iomeprol and Iopromide conformational equilibria in solution; then, the MD simulation and some experimental solution data extrapolated at infinite dilution are used to provide information about the hydration shell.

Second, spectroscopic and thermodynamic data are analyzed in order to describe the aggregation phenomenon and the dimension of aggregates by using some simple model, such as the isodesmic one. Additional information of the extent and the quality of aggregation is given by the results of MD simulations and calorimetric experiments, respectively. Then, some further structural information on the possible mechanism of the aggregation is taken form the temperature dependence of spectroscopic data, including the structural relaxation from Brilluoin spectroscopy.

Finally, the last chapters deal with the polymorphic forms of iopamidol and iomeprol in the solid state, by extending the characterization of the materials by means of diffractometric, calorimetric and spectroscopic methods. This investigation provides the rank of stability (obviously, in the solid packed form) of the several atropisomers, with a necessary correlation of the interaction equilibria in solution, described in the beginning of this section.
Results and Discussion

3.1. CHEMICAL SHIFT CHARACTERIZATION OF NICMs

To understand the molecular structure of NICMs studied in this work, a chemical shift characterization was performed. From the spectra the multi-signal of some proton and carbon nuclei suggested to probe atropisomerism via NMR. In the figure 3.1 spectra of the NICMs studied in this work are shown.

![Figure 3.1: (Left column) $^1$H-NMR spectra of Iopamidol, Iomeprol and Iopromide. (Right column) $^{13}$C-NMR spectra of Iopamidol, Iomeprol and Iopromide.](image)

By this study a chemical shift database of NICMs in aqueous solution was realized. Also the chemical shift of the precursors of Iomeprol were detected, but the values are explained in a next paragraph. In the table 3.1, 3.2 and 3.3 the chemical shift of the NICMs are summarized.
**Table 3.1** $^1$H and $^{13}$C chemical shifts of Iopamidol 0.5 M.
Values are referenced to internal TSP; values in parenthesis refer to the different forms.

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H Chemical Shift (ppm)</th>
<th>$^{13}$C Chemical Shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>144,7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>100,45</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>151,6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>91,6</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>151,6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>100,45</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>174,2</td>
</tr>
<tr>
<td>8</td>
<td>4,15</td>
<td>55,5</td>
</tr>
<tr>
<td>9</td>
<td>3,84</td>
<td>62,4</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>178,9</td>
</tr>
<tr>
<td>11</td>
<td>4,50; (4,75)</td>
<td>70,7; (70,7)</td>
</tr>
<tr>
<td>12</td>
<td>1,56; (1,57)</td>
<td>22,2; (22,2)</td>
</tr>
<tr>
<td>HN</td>
<td>8,99; 8,91</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.2** $^1$H and $^{13}$C chemical shifts of Iomeprol 1 M.
Values are referenced to internal TSP; values in parenthesis refer to the endo form.

<table>
<thead>
<tr>
<th>Position</th>
<th>$^1$H Chemical Shift (ppm)</th>
<th>$^{13}$C Chemical Shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>148,0; (150,5)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>100,2; (99,1)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>152,2; (151,7)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>92,9; (91,8)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>152,2; (151,7)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>100,2; (99,1)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>173,6</td>
</tr>
<tr>
<td>8</td>
<td>3,54; 3,41</td>
<td>44,5</td>
</tr>
<tr>
<td>9</td>
<td>4,01</td>
<td>71,9</td>
</tr>
<tr>
<td>10</td>
<td>3,74; 3,63</td>
<td>65,9</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>174,4; (174,9)</td>
</tr>
<tr>
<td>12</td>
<td>3,82; (4,53)</td>
<td>63,3; (62,5)</td>
</tr>
<tr>
<td>13</td>
<td>3,14; (3,22)</td>
<td>36,7; (36,9)</td>
</tr>
</tbody>
</table>
### Table 3.3 \(^1\)H and \(^{13}\)C chemical shifts of Iopromide 0.7 M.

Values are referenced to internal TSP; values in parenthesis refer to the different forms.

<table>
<thead>
<tr>
<th>Position</th>
<th>(^1)H Chemical Shift (ppm)</th>
<th>(^{13})C Chemical Shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>144.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>100.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>150.7</td>
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</tr>
<tr>
<td>4</td>
<td>91.6</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>151.9</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>100.3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>174.0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3.58; (3,44)</td>
<td>44.7</td>
</tr>
<tr>
<td>9</td>
<td>4.01</td>
<td>72.2</td>
</tr>
<tr>
<td>10</td>
<td>3.44</td>
<td>66.2</td>
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<tr>
<td>8’</td>
<td>3.75; (3,44)</td>
<td>52.7</td>
</tr>
<tr>
<td>9’</td>
<td>4.18</td>
<td>71.8</td>
</tr>
<tr>
<td>10’</td>
<td>3.64; (3,75)</td>
<td>66.4</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>174.4</td>
</tr>
<tr>
<td>12</td>
<td>4.27</td>
<td>73.7</td>
</tr>
<tr>
<td>13</td>
<td>3.60</td>
<td>62.5</td>
</tr>
<tr>
<td>14</td>
<td>3.21; (3,02; 2,97)</td>
<td>40.1</td>
</tr>
</tbody>
</table>

#### 3.2. DILUTE SOLUTIONS

##### 3.2.1. Atropisomerism

The \(^{13}\)C -NMR and \(^1\)H -NMR spectra of Iomeprol, iopamidol and Iopromide are complex to interpretate in terms of molecular conformation.

However, due to their intrinsically larger resolution, \(^{13}\)C-NMR spectra are much more informative. Virtually all different conformations are visible but difficult to distinguish. For this reason, assignment was accomplished by comparing \(^{13}\)C-NMR spectra of similar molecules with decreased complexity. The molecules analyzed in our work are shown in the Figure 3.2.
Results and Discussion

Figure 3.2: Compounds analyzed for the study of NICM. Compounds A, B, C and D are precursors. Compound E, F and G are Iopamidol, Iomeprol and Iopromide, respectively.

<table>
<thead>
<tr>
<th></th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>H</td>
<td>H</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>B</td>
<td>H</td>
<td>COCH₂OH</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>C</td>
<td>H</td>
<td>H</td>
<td>NHCH₂CHOHCH₂OH</td>
<td>NHCH₂CHOHCH₂OH</td>
</tr>
<tr>
<td>D</td>
<td>CH₃</td>
<td>COCH₂OH</td>
<td>NHCH₂CHOHCH₂OH</td>
<td>OH</td>
</tr>
<tr>
<td>E</td>
<td>H</td>
<td>COCHOHCH₃</td>
<td>NHCH(CH₂OH)₂</td>
<td>NHCH(CH₂OH)₂</td>
</tr>
<tr>
<td>F</td>
<td>CH₃</td>
<td>COCH₂OH</td>
<td>NHCH₂CHOHCH₂OH</td>
<td>NHCH₂CHOHCH₂OH</td>
</tr>
<tr>
<td>G</td>
<td>H</td>
<td>COCH₂OCH₃</td>
<td>NCH₂CH₂CHOHCH₂OH</td>
<td>NHCH₂CHOHCH₂OH</td>
</tr>
</tbody>
</table>

Figure 3.3 shows the spectral region of aromatic carbons in position 3, a region sensitive to conformational changes but also well resolved (similar effects are displayed by C1 and C5 not shown for clarity). Iodoftal (compound A figure 3.2) was chosen as the reference compounds for its simplicity (Fig. 3.3A). Atropisomerism is not present in this system.

**Syn/anti and E/Z equilibria**

The comparison of $^{13}$C spectrum of Iodoftal with compound C is able to unravel the effect of syn/anti and E/Z equilibria in these systems. In particular, the singlet belonging to carbons 3 and 5 is split into two signals with similar intensity (Fig 3.3C). Furthermore, a further splitting is present generating extra two peaks
Results and Discussion

with small intensity. The first splitting was attributed to the syn/anti equilibrium for the following reasons:

(i) E/Z conformers are rarely energetically equivalent and are unlikely to generate peaks with similar intensities;

(ii) IR spectra performed on Iopamidol (a close analogue) indicates a strong prevalence of E conformation in solution;

We can conclude that the syn and anti conformers are energetically similar, thus excluding significant interaction between the branches which could favour/disfavour one form with respect to the other.

Moreover, the further splitting observed in the peaks for compounds G (Iopromide) is due to the E/Z equilibrium with the E conformer largely predominating over the Z.

**SYN/ANTI and endo/exo equilibria**

The comparison of $^{13}\text{C}$ spectrum of Iodoftal with compound D provide a mean to get insight into the SYN/ANTI equilibrium not studied so far in the literature. As in the previous case, two equilibria (SYN/ANTI and exo/endo) generate two further splittings. In the spectrum of compound D, three sets of signals relative to C1,C3 and C5 are observed as C3 and C5 are not anymore equivalent. The larger splitting generating peaks with different intensity (Fig. 3.3D) is due to the exo/endo equilibrium. The assignment was verified in two ways:

(i) A splitting with similar population is also found in the $^1\text{H}$ spectrum and well evident for signals of the methyl in position 13. A 2D-$^1\text{H},^{13}\text{C}$ HOESY heteronuclear NOE cross peak between the methyl protons in position 13 and the carbon in position 12 was found only in the minor form wich is compatible with the endo conformer.

(ii) The value of the chemical shift of methylene 12 is expected to be significantly different in the exo and endo conformation. In the exo form these protons are placed on top of the shielding cloud of the
aromatic ring while in the endo form they point in opposite direction (see Figure 3.4). Accordingly, we found two forms at very different chemical shifts (3.82 and 4.53) whose intensity is larger for the exo form as compared to the endo. The figure also shows how the anisotropy of the carbonyl cannot affect significantly the chemical shift of protons in position 12 but does influence the shifts of aromatic carbons. In particular, as expected, C1 is deshielded while C3 and C5 are shielded in the less populated endo form.

The remaining splitting is consequently attributable to the SYN/ANTI equilibrium, generating forms with similar intensities. A confirmation of the assignment is obtained by 2D-\(^1\)H-NOESY spectrum in which only the amide proton of the branch in position 3 is visible. This proton is present in two similar populated forms, each displaying an NOE either with the methyl (as expected in the SYN form) or the methylene (as expected in the ANTI form) of the opposite anilido branch. Also in this case, the similar (although not identical) intensity of the peaks assigned to the SYN and ANTI forms suggests poor interaction between the branches.

Molecules like Iopamidol and Iopromide possess secondary anilido moieties which are not represented by the precursors analyzed so far. Figure 3.3B shows the \(^{13}\)C spectrum of compound B in which only the exo/endo equilibrium of such systems is present.

The figure shows only one peak for each carbon demonstrating that only one form is favoured or that the two forms are in fast exchange on the NMR timescale. IR measurements on Iopamidol revealed that all amidic bonds are in trans conformation, thus confirming the first hypothesis and assigning the main form to the trans conformer.
Figure 3.3: C3 peak multiplicity in iodinate compounds due to equiliria.
3.2.2. Equilibria in Iomeprol, Iopamidol and Iopromide

The analysis of the precursor has allowed to estimate the relative populations of the conformers in Iomeprol, Iopamidol and Iopromide (Table 3.4).

Table 3.4 Relative populations of the conformers in concentrated solution of Iomeprol, Iopamidol and Iopromide.

<table>
<thead>
<tr>
<th>Equilibrium/NICM</th>
<th>Iomeprol</th>
<th>Iopamidol</th>
<th>Iopromide</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYN:ANTI</td>
<td>50:50</td>
<td>not present</td>
<td>not present</td>
</tr>
<tr>
<td>syn:anti</td>
<td>33:67</td>
<td>29:71</td>
<td>38:62</td>
</tr>
<tr>
<td>endo:exo</td>
<td>23:77</td>
<td>not present</td>
<td>not present</td>
</tr>
<tr>
<td>E:Z</td>
<td>not present</td>
<td>not present</td>
<td>73:27</td>
</tr>
</tbody>
</table>
Before analyzing each compound, let us mention that the unpublished x-ray diffractometric data of this laboratory (R. De Zorzi, personal communication) on crystalline structures of anhydrous Iomeprol and Iopamidol pentahydrate show that in these molecules only the “anti” form is present, while the anhydrous Iopamidol crystallizes in the “syn” form. In addition, only the “exo” conformer is present in the crystal of Iomeprol, a form which is still the more abundant in the solution studies.

**Iopamidol**

The molecule is very similar to compound C but in position 1 has a secondary amine. Our study on precursors demonstrated that such kind of moiety is prevalently in trans (endo) conformation as also the remaining amides (vide supra). Moreover, Bradamante and al. demonstrated that there is free rotation along the C1-N bond\(^1\). In this respect Iopamidol displays only the atropisomerisms of compound C and two peaks are expected for each aromatic carbon due to the syn/anti equilibrium. Figure 3.3E shows that in this case one form is slightly preferred, probably due to the different geometry of the branches with respect to compound C. Steric hindrance considerations would suggest a larger population for the anti form (see Table 3.4).

**Iomeprol**

The molecule is quite complex, mixing the equilibria of compound C (syn/anti and E/Z) with those of compound D (SYN/ANTI and endo/exo). While we demonstrated that only E form is present in the branches in position 3 and 5, endo/exo splitting (Fig. 3.3F) is very evident in the spectrum (and similar to that observed in compound D). The relative populations of endo and exo forms is 23:77. Having solved the complexity due to the amidic bonds, we expect a further subdivisions of the peaks due to the SYN/ANTI and syn/anti equilibria. In particular, three main conformers should be present: SYNsyn, ANTIsyn and anti.

Accordingly, each peak generated by the exo/endo form is further spitted into three peaks with the more intense probably originating from the anti form (see Table 3.4).
Iopromide

Iopromide is definitively the most complex molecule in terms of NMR spectrum as different branches create many different signals, each complicated by many conformers. Three different amidic bonds are present although only one (in the branch bound to C3) is expected to display both E and Z forms. Due to the similarity of the anilido branch of Iopamidol, syn/anti equilibrium (but not SYN/ANTI) is expected to be present.

The aromatic region of the $^{13}$C spectrum (Fig. 3.3G) clearly shows a splitting in the region of C3, most likely due to the E/Z equilibrium introduced by the methylation of the amide in the branch. The assignment of the forms was based on the $^1$H shift of the methyl in position 14 which in the E form lies in the shielding cone of the aromatic ring and appear at higher chemical shifts. We could estimate a ratio of 73:27 for E and Z conformer, respectively. A further evident splitting is displayed in the $^1$H spectrum for the E form which might be due to the remaining equilibrium syn/anti. In this case however, a close inspection of the spectrum (both $^1$H and $^{13}$C) reveals many other further splittings making the complexity virtually unsolvable. The effect is probably due to the reduced symmetry of the molecule which is able to emphasize the effect of the stereochemistry of the two chiral branches which are present in racemic forms.

3.2.3. Molecular Dynamic simulations

To interpret thermodynamic parameters at the molecular level and to get a detailed description of both intramolecular and intermolecular interactions, molecular dynamics simulations were carried out. All simulations were physically carried out in the laboratory of Prof. JW Brady at Cornell University (USA).

The two main aspects that have been analyzed in detail were: the study of the hydration sphere of iopamidol and the interactions of iopamidol-iopamidol.

The first set of data is important to understand both the thermodynamic properties of dilute solutions and the experimental determination of the hydration volume calculated, for example, from data by hydrodynamic Stokes equation.
The second set of data provides crucial information to understand both the thermodynamic properties of concentrated solutions and the detailed description of the possible molecular interactions between solute molecules, in order to identify the moieties involved in the associative process.

The Molecular Dynamic simulations were carried out on the complete molecular structures of iopamidol. Furthermore, the simulations were carried out at different concentrations in order to study both the association and the hydration phenomena in terms of thermodynamic behavior of the system. The very diluted solution, but not infinitely as required from the thermodynamic, was approximated by a solute molecule in a cube containing 1273 water molecules (about 0.0044 m). The other two systems correspond to 0.5 m and 1 m solutions that were useful to carry out information on the solute-solute interactions.

The atomic coordinates of crystallographic iopamidol anhydrous[^2] reported by Ganazzoli et al. were used.

**Hydration**

The hydration analysis, on the iopamidol molecule in dilute solutions, was performed considering different trajectories. Two main types of information were taken into account: the number of water molecules contained in an arbitrary hydration volume and the interaction energy between the solvent and the solute molecules.

In the first case both the hydrophilic and the hydrophobic hydration molecules of water were included (see Figure 3.5a). The hydrophilic term corresponds to water molecules whose oxygen atoms are located within a distance of 3.4 Å from any polar atom of the solute as oxygen or nitrogen. The hydrophobic water molecules are those located within a distance of 4.5 Å from each non-polar atom as carbon or iodine. Or when a molecule of water is outside of 3.4 Å from each polar atom as oxygen or nitrogen.

Figure 3.5b shows an example of a trajectory of 1 ns. The instantaneous number of hydration value is shown as a function of time. From this graph both the frequency of the hydration number oscillation (of approx. 10 units) and the
decrease of the hydration number with time are clear. For this reason, other simulations were carried out to obtain an average hydration number at various concentration. These average results are shown in Table 3.5, where the water molecules are classified as hydrophilic or as hydrophobic.

![Figure 3.5](image)

**Figure 3.5**: (a) interaction energy of hydration waters and (b) total hydration number of Iopamidol calculated for 1 ns.

<table>
<thead>
<tr>
<th>Concentration (m)</th>
<th>Total number of water molecules</th>
<th>Hydrophilic water molecules</th>
<th>Hydrophobic water molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>51.2</td>
<td>20.8</td>
<td>30.4</td>
</tr>
<tr>
<td>0.5</td>
<td>42.2</td>
<td>17.9</td>
<td>24.3</td>
</tr>
<tr>
<td>1.0</td>
<td>36.5</td>
<td>16.0</td>
<td>20.5</td>
</tr>
</tbody>
</table>

The density fluctuations of the water molecules around Iopamidol were evaluated in order to identify the spatial arrangement of the solvent around the solute. A color code was used into a 3D (Figure 3.6) graph to distinguish the different probabilities of water concentration: the regions in red indicate an excess probability (high concentration of solvation water), while the lower probability is
shown in white. The radial distribution function is not symmetrical and reflects the configurational topology of hydrophobic and hydrophilic moieties.

Figure 3.6: distribution of the water molecule around Iopamidol.

Finally a comparison was made between the hydration numbers values obtained from these simulations and that derived by using the classical thermodynamic and rheological approach\textsuperscript{[3]}, suggested by Jones and Dole. The last approach assesses the hydration number as the difference between hydrodynamic volume (calculated from the Stokes equation) and the partial molar volume (calculated by density data). Density data from literature were analyzed to assess their quality and quantity for both Iopamidol\textsuperscript{[4]} and Iomeprol\textsuperscript{[5]}. Additional experiments from literature were found for both Iopamidol and Iomeprol\textsuperscript{[6]} at 37 °C.

The equation 3.1a was used to calculate the partial molar volume which is expressed as:

\[
\Phi V = d^{-1} (M_{NICM} + n_{water} \cdot M_{water} / m_{solute}) - n_{water} \cdot M_{water}/m_{solute} \quad 3.1a
\]
where $M_{\text{NICM}}$ is the molecular weight of the NICM, $n_{\text{water}}$, $m_{\text{solute}}$ are the water mole number in 1 kg of the pure water and the molality of the solute respectively. Thus, by using the literature density data, the apparent molar volume value can be calculated for Iopamidol and Iomeprol:

$$\Phi V = d^{-1} (777.1 + 55.51 \cdot 18.02/m) - 55.51 \cdot 18.02/m \quad 3.1b$$

Some autors did not give directly the experimental data but a best fit equation of their data. In these terms the quality of the data can not always be evaluated. The trend of $\Phi V$ as function of molality is shown in figure 3.7.

![Density values used:](image)

**Figure 3.7:** concentration dependence of apparent molar volumes of Iopamidol and Iomeprol from literature data.

The agreement between the data is quite poor and in some cases the trend has discontinuities, an evidence of a non-optimal data quality and clearly due to the limited number of significant digits. Unfortunately, it is impossible to extract any meaningful information from the trend of the data in the figure.
Therefore, as a different approach to evaluate the volumetric properties, all density data from the literature have been processed as specific density increments, in order to disclose possible data point deviation from the trend.

Interestingly enough, all the literature data points allign on a single polynomial curve (figure 3.8) with very little (expected) differences at high concentrations.

![Graph showing concentration dependence of specific density of both Iopamidol and Iomeprol at 25 °C and 37 °C (from literature data).](image)

**figure 3.8:** concentration dependence of specific density of both Iopamidol and Iomeprol at 25 °C and 37 °C (from literature data).

As the trend is the same for all the data, this overlap not only makes more easy to calculate average values of apparent molar volumes but also discloses a common value of the apparent and partial molar volumes of Iopamidol and Iomeprol at low concentrations. By using this approximation, the concentration dependence of the apparent molar volume at any temperature (20 °C < T < 37 °C) can be calculated (figure 3.9). Being the pure water density the only difference with the temperature, a small change also results in the apparent volumetric properties of Iopamidol with temperature when compared with the changes with the concentration.
The apparent molar volume of Iopamidol is thus described by the following equations:

\[
\Phi V = 397.34 - 66.70 m + 38.83 \ m^2 - 7.85 \ m^3 \quad (a \ 25^\circ C)
\]

\[
\Phi V = 399.30 - 67.03 m + 39.02 \ m^2 - 7.89 \ m^3 \quad (a \ 37^\circ C)
\]

The value given by the first term is defined by the relation:

\[
\lim_{m \to 0} \ \Phi V = \Phi V^\circ = V^\circ_2 \quad (at \ infinite \ dilution) \quad 3.2
\]

\[
V^\circ_2 \ (25 \ ^\circ C) = 397.34 \ \text{mL/mol}
\]

\[
V^\circ_2 \ (37 \ ^\circ C) = 399.30 \ \text{mL/mol}
\]

While density data provide the thermodynamic volume of the solute, experimental information on the hydration volume has been historically provided by calculating the hydrodynamic volume from viscosity data. The viscosity values, published in the literature by the same authors, were treated according to the equation:

\[
\log \eta_{rel} = A_3 c / (1 - Q'c) \quad 3.3
\]
the value of $A_3$ (for $c \rightarrow 0$) is related to the hydration volume through the equation:

$$V_h \approx \frac{2.303 \ A_3}{2.5} \approx 900 \ \text{mL/mol}$$

Therefore, from thermodynamic partial molar volume (397.34 mL/mol) and viscosimetric hydrodynamic volume (900 mL/mol), an average hydration number of Iopamidol, in a diluted solution, can be calculated:

$$\langle N_h \rangle \approx 28$$

This result should be considered with caution, because water molecules interact with different strength and mode with the solute and, furthermore, for “branched molecular species” as NICMs, the approximation of rigid spherical solute is broken. However, even with the limits of this approximation, the agreement between the results from molecular dynamics and the results from processing thermodynamic and rheological data is satisfactory.

### 3.3. EFFECT OF CONCENTRATION AND STRUCTURAL FEATURES OF AGGREGATES

#### 3.3.1. Evidence for a concentration effect in NMR spectra

A remarkable effect observed in $^{13}$C spectra performed at moderate concentrations (larger than 0.4 M) is the doubling of some peaks. Figure 3.10 reports the effect of concentration on the $^{13}$C aromatic region of Iomeprol and Iopamidol showing that the peak assigned to the “anti” form (i.e. the most abundant conformer for both molecular species) splits at high concentrations. The most simple explanation is that this splitting is due to some new intermolecular interactions arising at high concentration. Which group is involved cannot be simply resolved in a NOESY experiment, since it is not possible to distinguish between intra and intermolecular NOE in a concentrated solution of each NICM.

As a strategy for solving the problem, including the intermolecular interactions involving the branches, 2D-NOESY spectra were recorded on a 1.1
mixture of Iomeprol and Iopamidol. Due to the similarity of the molecules it is assumed that the mixture is a good model to monitor intermolecular interaction involving the branches. Interestingly both H8 and H9 of Iopamidol display NOEs with the same protons (and also with the methyl H13) in Iomeprol, an effect compatible with the pairing of the branches in 3, 5 observed in both the crystals (Figure 3.11). Finally, a further NOE between methyl 12 in Iopamidol with methyl 13 in Iomeprol seems to reproduce the relative orientation of the Iomeprol molecules in the crystal.

![Figure 3.10: Concentration effect of 13C spectra of Iopamidol and Iomeprol. Doubling of peaks is evident (vertical diverging arrows) at high concentrations. Horizontal arrows indicates splitting due to syn/anti and exo/endo equilibria in Iopamidol and Iomeprol, respectively.](image)

The results of the solution conformation and interactions of iodinated contrast media provide some insight into the structural features of the soluble aggregates in concentrated solutions (at concentration used for clinical purposes). Out of the many possible atropisomers, only few are significantly populated, most likely the same found in their parent crystal structure. Furthermore, the conformational analysis indicates the presence of a sterically hindered amidic
bond, allowing a significant population of cis form (Z in Iopromide and exo in Iomeprol), that may assist the slightly larger solubility of these compounds as compared to Iopamidol.

![Figure 3.11](image)

Figure 3.11: (left) NOE contacts in a mixture of Iomeprol and Iopamidol. (right) Crystal structure of Iopamidol pentahydrate.

3.4. FTIR-ATR

Experiments as a function of concentration were done to probe IR shift changes. Figure 3.12 shows the overlapped spectra of Iopamidol. Their correction was performed by subtraction of the water profile measured at 25 °C. It is important to consider that the resulting amide I signal is heavily superimposed to the water bending mode. Thus the subtraction implies that the amide I stretching signal does not change (i.e. water structure is poorly affected by the solute). (Figure 3.12) The overlapping spectra show also very small changes with concentration in terms of band shift, overall shape and relative intensity. No drastically changes were detected into the explored concentration range except the
Results and Discussion

peak positions of the amide signals (Figure 3.12) that show some variation with concentration.

![Figure 3.12: Overlapping IR spectra of Iopamidol at different concentration.](image)

![Figure 3.13: graphs of frequency shift vs concentration for Iopamidol solution.](image)
The variation is rather regular and indicates a kind of continuous changing in the solvation properties of the solute as shown in the Figure 3.13:

All the peaks shift to lower frequencies and this is particularly evident for the amide II signals which show a significant shift to lower frequencies with concentration. For these bands, a similar red-shift occurs also by increasing temperature; the common interpretation is that upon increasing temperature or concentration the NH group becomes less involved in hydrogen bonds with the solvent. Under the same circumstances, also the amide I slightly shifts to lower frequencies and the phenomenon is similar to a temperature decrease. The interpretation is an increase of both dipolar and other solute-solute interactions at higher concentration, mostly independent of the H-bonding situation that exists around the C=O moiety.

**Perturbation of the water organization**

Decreasing temperature in a sample of pure water, the hydrogen bonds are strengthened and a blue-shift of the combination band can be observed. From the water point of view, the Figure 3.14 shows that there are not significant changes in the combination band mode of the water.

*Figure 3.14:* combination band at different concentration of water after subtraction of pure water profile.
From the water point of view, Figure n, the self similarity of amide I peaks implicitly implies that the bending mode is poorly affected by the solute. The good compensation in the combination region supports this fact. In any case a small effect is detected which is indicative of an increase of the intermolecular potential felt by water molecules.

3.5. SELF-ASSEMBLING: ISODEMIC MODEL

3.5.1. Premise

The process of molecular association induces local and global changes to the molecules involved, which alter their physico-chemical behaviour in solution. The intermolecular hydrophobic and hydrophilic interactions have been found to govern different supramolecular nano-organizations. No common model has been developed to interpret the experimental observations. Therefore, a scrutiny study on the NICM systems is necessary to understand both the role and the nature of the noncovalent intermolecular interactions involved.

In this part of the work, the concentration dependences of $^1$H chemical shifts of the NICMs have been investigated and analyzed, for a quantitative determination of the self-association equilibrium. Similarly, by using osmolality data from literature, the self-association constants were calculated. In both cases, the simple “isodesmic model” was used. The isodesmic model of solute self-association is based on the assumption that solute molecules associate to form dimers, trimers, etc., with an equilibrium constant $K$ equal for each step.

Thus, for any value of $j= 1…n$:

$$A_j + A \rightleftharpoons K \rightarrow A_{j+1}$$

At any stage of aggregation, the sum of all associated species contributes to the nominal concentration in the form:

$$[C_t] = [A_1] + [A_2] + 2[A_3] + ... n[A_n] + ...$$
where \([C_t]\) is the total concentration of solute A.

The explicit form of the series expansion is:

\[
[C_t] = \frac{[C]}{(1 - K[C])^3}
\]

\([C] = [A_1]\) is the concentration of the monomer in solution.

The process is considered as an addition of one monomer per time, but no assumption is made about whether the mechanism goes via the association of one oligomeric species at the time or by the condensation of already formed aggregates. At the macro level this type of open polymerization generates at equilibrium a series of polymers having a continuous distribution of molecular weights.

### 3.5.2. Self-assembling by NMR

In order to localize the regions for intermolecular interactions the effect of concentration on chemical shift has been investigated by using samples of each contrast agent ranging from 0.008 mM to 1 M (Figure 3.4.2). Chemical shift differences were referenced to internal TSP in ratio 1:100 in each sample. Deviations from the values of the least concentrated sample are shown in Figure 3.15.

Largest shifts were mapped onto the structure (Figure 3.15, bottom) to localize the region of intermolecular interaction. Carbonyl (C7 and C11) and amide protons in the branches display the largest effect excluding a major role of aromatic ring stacking in the formation of aggregates. Taking into account the limitations in the observable protons (e.g., no OH proton is observable), these results seem indicate that amido groups are involved in the intermolecular interactions.
Figure 3.15: (top) Concentration dependence of $^1$H and $^{13}$C chemical shift of Iomeprol, Iopamidol and Iopromide relative to their value at 0.008M and 0.2M, respectively. 
(bottom) Molecular topology of chemical shift changes on concentration. Larger shifts are displayed as lighter colours. White is used for atoms whose shifts was not measured.
Equilibrium constant and number of particles

Chemical shift data as a function of concentration can provide the values of the self-association constant and the average number of particles per aggregate\(^7\) by using the isodesmic model\(^8\). The value of the association constant for each step of the aggregation (from monomer to dimer, from dimer to trimer and so on) is obtained using the equation\(^9\):

\[
\delta_{\text{obs}} = \delta^m + \Delta_0 K[C][2 - K[C]]
\]

\[
[C] = C_t \left[ \frac{2}{1 + \sqrt{4KC + 1}} \right]^2
\]

where \(\delta_m\) is the chemical shift of the monomer, \(\Delta_0\) is the limiting deviation of the aggregate from \(\delta_m\) (=\(\delta_{\text{aggr}}\)-\(\delta_m\)), \([C]\) is the equilibrium concentration of the monomer and \(C_t (=\Sigma n_i C_i)\) the total concentration of the species.

Following the chemical shift of the amide proton we obtained values of \(K=0.3 \pm 0.5\) M\(^{-1}\) (Iomeprol), \(K=0.2 \pm 0.5\) M\(^{-1}\) (Iopamidol), and \(K=0.4 \pm 0.5\) M\(^{-1}\) (Iopromide). The fitting also provide the limiting chemical shift value of the aggregate \(\delta_{\text{aggr}}\).

As stated above, the value obtained for \(K\) cannot be interpreted as the global association constant if the actual number of molecules per aggregate \(n\) is unknown. An estimate of such number can be provided by the same data, plotting \(\ln(C\Delta)\) as a function of \(\ln(C\Delta_0)\) and fitting by the following curve\(^{10}\):

\[
\ln(C\Delta) = n \ln(C\Delta_0) + \ln K_a + \ln(n) - (n - 1) \ln \Delta_0
\]

where \(\Delta\) is the observed deviation from the shift of the monomer (=\(\delta_{\text{obs}}\)-\(\delta_m\)) (figure 3.15, upper panel). The method allowed a refinement of the values for the association constants (\(K=0.31\) M\(^{-1}\), \(K=0.15\) M\(^{-1}\) and \(K=0.24\) M\(^{-1}\) for Iomeprol, Iopamidol, and Iopromide respectively). The values found are approximately ten times smaller than those found by osmolarity (see next section). However, here
aggregation is monitored by using a specific probe, i.e. the amide proton chemical shift. Only specific interactions will give rise to significant deviations in chemical shift are taken into account. Therefore, other interacting species may involve groups that are not monitored.

3.5.3 Self-assembling by thermodynamic data

Osmolality

Results of osmolality experiments performed at 37 °C and reported in literature provide other data to check the association and compare the trend of osmotic coefficients of Iopamidol\(^4\) and Iomeprol\(^5\). The osmotic coefficient of all the two NICMs indicates the presence of association phenomena that depend from concentration. In the figure 3.16 osmotic coefficients vs concentration of Iopamidol, Iomeprol and Iopromide respectively are shown, together with the polynomial fitting equation.

![Figure 3.16: osmotic coefficients of Iopamidol and iomeprol in aqueous solution as a function of molality](image)

Self association constants can be calculated from osmolality data in the molality scale by using procedures already reported in literature. As for the NMR studies, the association process was considered in terms of an isodesmic model.

It is possible define the osmotic coefficient in the follow way (see for example ref [11]):
where \( m \) is the molality concentration without associative processes and “\( \tilde{m} \)” is the effective molality that considers the presence of associated species in solution (osmolality).

So, the osmotic coefficient can be calculated as the ratio of the two concentrations above reported

\[
\Phi = \frac{\tilde{m}}{m}
\]

\( \Phi \) is an effective measure of the deviation from the ideality system. In absence of any other non-ideality phenomenon, it takes fully into account an associative process of the species in solution (see caffeine). When hydrophobic solutes are involved, the ideality of the solution can also be affected by the extent of hydration water can be significantly different from the bulk water in terms of thermodynamic activity. Thus, correct evaluation of association constants needs to consider hydration processes also.

In general, given the concentration of the oligomeric species in the association equilibrium, the definitions above written allow to calculate the osmotic coefficient. This fact lets to consider another simplifying approximation assuming the same variation of the thermodynamic properties for every step of association process. So, every reaction step is characterized by the same value of \( \Delta G \), \( \Delta H \) and \( \Delta S \) and it is possible write:

\[
1 - \Phi = Km\Phi^2
\]

where \( K \) is the association constant that is the same for each association step:

\[
K = K_1 = K_2 = K_3 = K_4 = \ldots
\]
In the equation only $K$ is unknown:

$$K = \frac{1 - \Phi}{m\Phi^2} \quad 3.10$$

The association constant for Iopamidol and Iomepril calculated are $1.6 \text{ m}^{-1}$ and $3 \text{ m}^{-1}$ respectively.

### 3.6. DIFFUSION AND RELAXATION TIMES

#### 3.6.1. Diffusion coefficient

In order to better characterize the size of these aggregates we performed a series of DOSY experiment to measure the diffusion coefficient and eventually the hydrodynamic radius. Data are reported in table 3.6.

<table>
<thead>
<tr>
<th>Contrast media concentration, (M)</th>
<th>Diffusion Iomepril, $(x10^{-11}) \text{ m}^2/\text{s}$</th>
<th>Diffusion Iopamidol, $(x10^{-11}) \text{ m}^2/\text{s}$</th>
<th>Diffusion Iopromide, $(x10^{-11}) \text{ m}^2/\text{s}$</th>
<th>Viscosity of Iomepril sol. $(x10^3) \text{ Kg/(m.s)}$</th>
<th>Viscosity of Iopamidol sol. $(x10^3) \text{ Kg/(m.s)}$</th>
<th>Viscosity of Iopromide sol. $(x10^3) \text{ Kg/(m.s)}$</th>
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<td>0.08</td>
<td>44.72</td>
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<td>0.90</td>
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<td>9.37</td>
<td>4.50</td>
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<td>6.24</td>
</tr>
</tbody>
</table>

*Values for TSP at 0.01 M and $8x10^{-5}$ M in water were $78.70 \times 10^{-11} \text{ m}^2/\text{s}$ and $82.81 \times 10^{-11} \text{ m}^2/\text{s}$, respectively. Their similar values demonstrate the absence of aggregation phenomena which might change the assumed hydrodynamic radius of TSP introducing a bias in the determination of the viscosity (see text).

** Actual concentration was 0.87 M
The diffusion coefficient $D$ is dependent on the value of the viscosity of the solvent, according to the Stokes-Einstein relation\cite{12} \cite{Cavanagh J, Fairbrother WJ, Palmer III AG et al. Protein NMR Spectroscopy. Principles and practice. 1996; Academic press, Inc]:

$$D = \frac{k_B T}{f_T} \quad f_T = 6\pi\eta r_H$$

3.11

where $f_T$ is the friction factor, $k_B$ is the Boltzmann constant, $\eta$ is the viscosity of the solvent and $r_H$ is the hydrodynamic radius of the molecule, here assumed of spherical shape. In cases where the the solute is concentrated as in our case, the viscosity (and accordingly the diffusion coefficient) becomes non trivially dependent on concentration\cite{10}. For this reason, the diffusion coefficient $D$ of internal TSP was used to derive the viscosity of each solution (reported in table 3.6), to be used for determining the hydrodynamic radius of iodinated contrast media in each sample. Given the measured diffusion coefficient and the hydrodynamic radius of TSP in water ($0.351$ nm\cite{13}), the viscosity of the solution is calculated by using equation 3.11.

Despite the quite different chemical-physical properties of TSP with respect to the studied molecules, the method allowed to estimate quite precisely the molecular weight of the contrast agent monomers at low concentration. Furthermore, the similar values of the diffusion coefficient found for TSP in water at the maximum and minimum concentrations used in our experiments and in the absence of contrast media (see note in table 3.6) demonstrate that the molecule is not prone to aggregation (which would result in an error in the determined viscosity). The values of viscosities were used in 3.11 to measure hydrodynamic radius $r_H$ of aggregates from experimental diffusion coefficients in each solution (figure 3.17).
Figure 3.17. (Upper panel) Equilibrium constant and number of particles per aggregate in Iomeprol, Iopamidol and Iopromide; (Lower panel) Effect of concentration on hydrodynamic radius and molecular weight of aggregates.

In order to roughly estimate the molecular weight of the species, the apparent molecular weight $M$ was calculated$^{[14],[15]}$. 
where $N_A$ is Avogadro’s number, $\nu_2$ and $\nu_1$ are the partial specific volumes of the molecule and solvent water, respectively, and $\delta_1$ is the fractional amount of water bound to the molecule (hydration number). $F$ is the shape factor, or Perrin factor, which is defined to be the ratio of the friction coefficient of the molecule to that of a hard sphere with equivalent mass and partial specific volume. We considered an axial ratio of 1 ($F=1$) because the single molecule is on average a sphere as the branches are pointed orthogonally to the aromatic plane to avoid the hindrance with Iodine atoms. As for the values of the partial specific volumes $0.36 \times 10^{-6}$ m$^3$g$^{-1}$ and $1 \times 10^{-6}$ m$^3$g$^{-1}$ were used for contrast media and the solvent. The first value is the half of the value generally used for biological molecules$^{[12]}$ ($0.73 \times 10^{-6}$ m$^3$g$^{-1}$) because of the presence of three iodine atoms which alone account for the half of the molecular weight. For the same reason a value of 0.15 was used for the hydration number (hydration numbers in the range 0.3–0.4 gram of water per gram of molecule are common for most biological molecules$^{[15]}$). The apparent molecular weight from 3.12 was found at 766 for the most dilute solution in remarkable agreement with the molecular weight of Iomeprol (777) even if at very low concentrations, viscosity values are less accurate due to a worst signal/noise ratio affecting the precision of measured diffusion coefficients. Also the hydrodynamic radius (539 pm) is reasonable for such molecule.

Figure 3.17 (lower panel) shows how both the hydrodynamic radius and the apparent molecular weight increase with the concentration. While the increase of the radius is linear the molecular weight increases exponentially (because is proportional to the volume of the sphere representing the aggregate). The deviation from linear behavior of the hydrodynamic radius between 0.8 and 1 M may be due to some aggregation of TSP with Iomeprol which could affect the measured viscosity at 1M.

Actually, the values of viscosity found are in substantial agreement with data in literature$^{[5]}$ but differ at the highest concentration. Focusing ion what observed at 0.8 we find a molecular weight of 2639 for Iomeprol and 1771 for Iopamidol. These values suggest an average of 3.4 and 2.3 molecules per
aggregate, respectively. However, care should be taken in the interpretation because the values also depend on the shape of the aggregates which was assumed spherical.

### 3.6.2. Relaxation time

Relaxation measurements are able to probe molecular motion of nuclei. The presence of intermolecular interaction can interfere with the mobility of molecules thus localizing the regions of contact. Both longitudinal and transversal relaxation rates ($R_1$ and $R_2$) of $^1\text{H}$ and $R_1$ of $^{13}\text{C}$ of Iomeprol, Iopamidol and Iopromide were carried out at different concentrations (tables 3.7a, b and c).

The main mechanism for proton relaxation is due to the dipolar interaction among protons. The relaxation rate depends on local motions (described by the correlation time $\tau_c$) and inter-proton distances. Due to the flexibility of the molecule the latter parameter (which is extremely important due to the six power dependence) is not measurable in our case.

However, in absence of chemical exchange, the ratio between $R_1$ and $R_2$ due to dipolar interaction, is practically only dependent on the correlation time, according to the following relation\textsuperscript{[16]} (3.13):

$$\frac{R_1}{R_2} = \frac{\frac{6\tau_c}{(1 + \omega_H^2 \tau_c^2)} + \frac{24\tau_c}{(1 + 4\omega_H^2 \tau_c^2)}}{\frac{15\tau_c}{(1 + \omega_H^2 \tau_c^2)} + \frac{6\tau_c}{(1 + 4\omega_H^2 \tau_c^2)} + 9\tau_c} \quad 3.13$$

where $\omega_H$ is the proton Larmor frequency and $\tau_c$ is the correlation time modulating the dipolar interaction.
### Table 3.7a

$^1$H R$_1$ (s$^{-1}$) measurements for Iomeprol, Iopamidol and Iopromide at different concentrations

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Equation 3.13 was used to derive motional correlation times for all protons in Iomeprol, Iopamidol and Iopromide at different concentrations (figure 3.18).

| 1H R$_2$ (s$^{-1}$) measurements for Iomeprol, Iopamidol and Iopromide at different concentrations |

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Results and Discussion

Figure 3.18. (upper panel) Effect of concentration on mobility as monitored by $^1$H relaxation; (central panel) effect of concentration on $^{13}$C $R_1$; (bottom panel) effect of concentration on mobility as monitored by $^{13}$C relaxation.
Results and Discussion

Figure 3.18 (upper panel) clearly shows how especially in iomeprol the motion of protons nearby amidic bonds in the branches slow down with concentration (increase in motional correlation time), confirming what observed by chemical shift drifts (figure 3.15). The effect is not due to viscosity because in this case all protons would behaved in the same way. The small effect due to viscosity is monitored by TSP whose motion is almost constant. The higher value obtained for protons 8 and 9 is probably an artefact due to spin-rotational mechanism not considered here. However, the constant value found for 9 and 10 seems to exclude this region of the molecule in the aggregation process in iomeprol. In addition, no significant difference is observed for the endo (12’, 13’) and exo conformers.

Iopamidol displays much smaller effect, in agreement with chemical shift and diffusion data. Mobility was monitored also by $^{13}$C relaxation (table 3.7c) to probe motions on the aromatic ring and derive the global motional correlation time of molecule.

In the case of carbons directly attached to protons, relaxation is mainly due to dipolar interaction
For carbon atoms not directly attached to protons the main relaxation mechanism is Chemical Shift anisotropy (CSA)\cite{12}:

$$
R_{1}^{CSA} = \frac{2}{5} \left( \frac{\omega_{c}\Delta\sigma}{\sqrt{3}} \right)^2 \left\{ \frac{\tau_{c}}{1 + \omega_{c}^2 \tau_{c}^2} \right\}
$$

3.14

where $\Delta\sigma$ is the chemical shift tensor anisotropy in ppm whose value is needed to extract the correlation time from equation 3.14.

Figure 3.18 (central panel) shows the trend of relaxation rate for each carbon. It is clear that all carbons attached to protons increase their $R_1$ with concentration while all the others, governed by CSA relaxation, reach a maximum and then decrease. By plotting equation 3.14 for different values of $\Delta\sigma$ it was possible to reproduce the maximum of each curve thus deriving the exact value of $\Delta\sigma$ reported in Table 3.7c.
Equation 3.14 was used to estimate the correlation times of all aromatic and carbonyl carbons (Figure 3.18, bottom panel). Data show how aromatic carbons behave as a whole with carbonyls of the branches, demonstrating that indeed the rotation about the C-aryl and C-N bond is much slower than molecular tumbling (but fast for Iomeprol in the NMR acquisition time scale as demonstrated by chemical shift data). All part of the molecule tends to decrease the motion with increasing concentration due to formation of aggregates. Also in this case the effect is less pronounced for Iopamidol.

In order to demonstrated that the observed showing of the motions is not due to viscosity, we calculated the correlation time expected at different concentrations for a rigid sphere having the molecular weight of a monomer. The calculated values were compared with the experimental ones (using the average correlation time found for aromatic and carbonyl carbons at each concentration).

The approximation of a rigid rotor is a good model because we consider only the aromatic and carbonyl carbons, for which internal mobility is negligible. The global correlation time of a rotor having a defined molecular weight can be estimate by using the Stokes-Einstein equation\[^{[12]}\]:

\[
\tau_c = \frac{4\pi \eta r_H^3}{3k_BT}
\]

with:

\[
r_H = \sqrt{\frac{3\nu_2 MW}{4\pi N_A} + r_w}
\]

where \(r_H\) is the hydrodynamic radius, \(k_B\) is the Boltzmann constant, \(T\) is the absolute temperature, \(\eta\) is the viscosity of the solution, \(N_A\) is Avogadro’s number, \(\nu_2\) is the partial specific volume of the molecule and \(r_w\) is the solvent radius\[^{[12]}\] (between 0.16 and 0.32 nm) corresponding to one-half and one hydration shell; we used the lower value as in the diffusion data interpretation).
### Table 3.7c $^{13}$C R$_1$ (s$^{-1}$) measurements for Iomeprol, Iopamidol and Iopromide at different concentrations

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<td>4.37</td>
<td>4.04</td>
<td>3.92</td>
<td>3.77</td>
<td>3.52</td>
<td>3.10</td>
</tr>
</tbody>
</table>

Results and Discussion
By using equations 3.15 and 3.16 (with the viscosity previously measured by DOSY) we calculated the correlation time expected for a monomer at different concentrations (figure 3.19) and compared the values with the averages found in Iomeprol. The figure shows that, even though the viscosity do influence the measurements, the observed slowing of the motion is due to a different phenomenon such as aggregation.

![Figure 3.19 Global correlation times as a function of Iomeprol concentration.](image)

Experimental values are obtained by averaging the correlation times for aromatic and carbonyl carbons derived from relaxation measurements, calculated values are obtained from equation 3.15 and 3.16 (monomer) or 10, 11 and 12 (aggregate) using viscosities found by diffusion measurements. In the table 3.8 the results for concentrated solutions (0.8 M) are shown.

<table>
<thead>
<tr>
<th>NICM</th>
<th>MW (g/mol)</th>
<th>(r_H) (pm)</th>
<th>Conc. (mol/L)</th>
<th>Average Number of molecules per aggregate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iomeprol</td>
<td>2639</td>
<td>813</td>
<td>0.8</td>
<td>3.4</td>
</tr>
<tr>
<td>Iopamidol</td>
<td>1771</td>
<td>712</td>
<td>0.8</td>
<td>2.3</td>
</tr>
<tr>
<td>Iopromide</td>
<td>2719</td>
<td>822</td>
<td>0.8</td>
<td>3.4</td>
</tr>
</tbody>
</table>
3.7. MOLECULAR DYNAMIC SIMULATIONS (ASSOCIATION)

The concentration values of Iopamidol were 0.5 m and 1 m and were analyzed to evaluate the self assembling parameters. The simulations show a strong tendency to form molecular clusters whose dimensions are nanoscopic, even if the self-assembly is floating. A snapshot of this situation is provided in Figures 3.20 and 3.21, which show two examples of extended self-assembly of iopamidol at a concentration of 0.5 and 1 m.

Figure 3.20: cluster of Iopamidolo at 0.5 m.

Figure 3.21: cluster of Iopamidol at 1 m.
The statistic analysis of self assembling data were calculated as an average of simulations performed to 1 ns to 5 ns and 10 ns. These simulations shown the size increase of the nano-structured aggregate with time. These simulations shown also some molecules in the aggregate can be solved by water and to leave the soluble supramolecular structure.

3.8. CALORIMETRY ON THE SOLUTIONS

3.8.1. Heats of dilution

The heats of dilution of the NICM solutions were carried out to obtain the graphs of apparent molar enthalpy in function of concentration. The main results of Iomeprol, Iopamidol and Iopromide are shown in the Figure 3.22.

![Figure 3.22: heats of dilution of Iomeprol, Iopamidol and Iopromide.](image-url)
Iopamidol

The dilution of iopamidol was exothermic (negative $\Delta H_{\text{dil}}$) even if the amount of heat involved is relatively small, it is between 2-3 kJ mol$^{-1}$ for the complete process of dilution from high dilution at infinite dilution. This fact, given the molecular complexity of the system, must necessarily lead to the conclusion that the total heat of dilution is the sum of a large number of both types polar and non-polar contributes: the different amide groups and hydroxyl for the first case and the aromatic group that attaches three iodine atoms for the second case. From the enthalpy balance is clear that it indicates a prevalence of non-polar interactions in the dilution process. Furthermore, the results indicate that the heat generated in the process of dilution decreases with temperature. The measurements were carried out at 25 °C and 37 °C. These data were carried out by two series of dilution using two vials as received demonstrating both the reproducibility of the measures and the quality of the data.

Iomeprol

The dilution of Iomeprol was endothermic (positive $\Delta H_{\text{dil}}$) and, as for Iopamidol, also Iomeprol shows an amount of heat involved in the dilution process relatively small and its value is between -1 and -2,5 kJ mol$^{-1}$ in all measurement range. As in iopamidol system also here the total heat of dilution is the sum of a large number of both polar and non-polar interactions. But for Iomeprol the enthalpy balance shows a prevalence of polar interaction during the dilution process measured by two series of measures using two vials as received. The measures were performed at 25 °C and 37 °C that show the dependence of the heat of dilution: it decreases with temperature.

Iopromide

The behaviour of Iopromide during the dilution process is similar to that of Iopamidol. The exothermic process, during the dilution of concentrated solution, demonstrates that the non-polar interactions are prevalent. The heat of dilution involved into the dilution process is, also here, relatively small and its value is between 1,5 and 2,5 kJ mol$^{-1}$. Iopromide shows a prevalence of non-polar
interactions during the dilution process and the heat involved decreases with temperature. The experiments were carried out at 25 °C and 37 °C. For every temperature the curve was obtained by two series of measurements.

NICMs compared

The thermal process during the dilution is relatively small. The average value of the heat measured does not exceed 2-3 kJ mol\(^{-1}\). The apparent molar enthalpy of Iomeprol is, in terms of absolute value, slightly lower than that both Iopamidol and Iopromide in all explored concentration range (Figure 3.22).

However, the most important fact is that the dilution of Iopamidol and Iopromide is an exothermic process, while the dilution of Iomeprol is endothermic. This marked difference in behavior is maintained even with changes in temperature. Increasing temperature is always related with a decrease of apparent molar enthalpy.

The different enthalpy behavior represents an important correlation "structure-property" in terms of both chemistry and commercial kind.

From a thermodynamic point view the prevalence of non-polar interactions in Iopamidol and Iopromide should lead to the conclusion that the mechanism of aggregation should be based, in part, on a solvophobic process type considering the coefficient osmotic values shown below. It means that the aggregation in solution is not mediated exclusively by direct solute-solute interactions.

3.8.2. Heat capacity and apparent specific heat

The specific heat provides an accurate determination of changes in enthalpy (and entropy) of the system as function of temperature. Energetic changes of both pure and mixed systems can be carried out due to the utmost sensitivity for the detection. The concentration dependence studies are performed at four temperatures: 25, 29, 33 and 37°C. The results of Iopamidol are summarized in the Figure 3.23:
Four polynomial regressions were used for each temperature. Correction on the base line was not done and this could explain the trend of the points at 0.6 m. The apparent specific heat of Iopamidol ($\Phi C_p / J K^{-1} g^{-1}$) increases with temperature and significantly decreases with concentration. The absolute $\Phi C_p$ value is small enough (between 1.3 and 1.4 JK$^{-1}$g$^{-1}$) if compared to that of the water (4.18 J K$^{-1}$ g$^{-1}$ at 15°C).

Similarly, also for Iomeprol its specific heats were carried out at the same temperature values (25, 29, 33 and 37°C). In the figure 3.23 the results of Iomeprol are shown compared to those Iopamidol. The comparison between heat capacities (figure 3.23) shows a heat capacity contribution of Iomeprol less than that of Iopamidol at high concentration. Furthermore, Iopamidol shows an highest $\Phi C_p$ value in all concentration range probed. Furthermore, its $\Phi C_p$ shows also, at isothermal conditions under 0.6 m, the $\Phi C_p$ a concentration dependence. Iomeprol does not show the same behaviour.
3.9. EFFECT OF TEMPERATURE

3.9.1. Temperature dependence of NMR chemical shift

To study the formation and the nature of hydrogen bonds in solution by $^1$H-NMR spectroscopy, an experimental method was found in literature\cite{17}. This study, performed in function of temperature, was achieved by monitoring the changes in the amide proton chemical shift of Iomeprol. The NMR does not distinguish the free form and the bonded form in a fast exchange, it can see only an average value. In Iomeprol there are two types of amide bonds, but in its $^1$H-NMR spectra three signals of NH moiety appear due to the atropisomerism phenomenon. The assignment for each NH signal to the atropisomers was not possible. In figure 3.24 the results are shown:

![Figure 3.24 : Chemical shift vs temperature on Iomeprol solution 1 M.](image)

In the graphs are reported chemical shift and temperature. The linear trend of the data was fitted by a linear fit characterized by a negative slope value. If the slope value is less negative than -4.5 ppb/K it means that the moiety is involved in hydrogen bonds with the solute\cite{17}. While, if the slope value is more negative than -4.5 ppb/K the system is not involved in hydrogen bonds with the solute, but the amidic bond could be interact with the solvent. By this experiments the slope
value obtained were: -9.2 ppb/K, -7.5 ppb/K and -8 ppb/K. Being the slope values more negative than -4.5 ppb/K it means that amidic bonds are not involved in hydrogen bonds with the solute. To probe the nature of hydrogen bond of the amidic group with the water, Iomeprol was analyzed by IR-ATR method.

3.9.2. Temperature dependence of IR-ATR band shift

The IR-ATR measurements were carried out at five temperatures on Iopamidol vials solution: 25 °C, 35 °C, 42 °C, 55 °C and 67 °C. Each spectrum of Iopamidol was corrected by subtracting the spectrum of pure water at the same temperature (Figure 3.25) rescaled on the water combination band (Figure 3.26).

The great compensation of the water combination band indicates a small effect on the water hydrogen bonding induced by Iopamidol. In fact this band, in general, does not show very strong variations also in other related systems (see acetone) when a change is expected. In this case a minor effect at lower temperature is detectable and it is consistent with an eventual strengthening of the water potential.

![Figure 3.25: combination mode bands of pure water at different temperature.](image-url)
Results and Discussion

Figure 3.26: difference spectra realized by subtracting the spectra of pure water at the same temperature rescaled on the water combination band.

The overlapping spectra, shown in Figure 3.27, was made to assess the peak shift with temperature.

Figure 3.27: overlapping spectra of Iopamidol solution concentrated at different temperature.
Results and Discussion

The band positions are consistent with a trans configuration of the amide groups. This is confirmed by the high frequency region (overtone at 3060 cm\(^{-1}\) and NH stretching at 3210 cm\(^{-1}\))\(^{[18]}\). The shift of the bands are shown in the Figure 3.28:

![Figure 3.28: shift of the amide bands.](image)

The increase of temperature causes a blue shift of the amide I, a C=O stretching mode. When the carbonyl moiety is involved in an hydrogen bond, its stretching frequency decreases because the bond between oxygen and carbon is less strong. When the hydrogen bond is broken, the double bond of C=O becomes more strong. The consequence is an increase of its stretching frequency that suggests a breaking of an hydrogen bond. The band of amide II, a NH bending mode, shows a red shift with temperature. This result suggests that NH moiety forms a hydrogen bond and it is explained considering that the hydrogen bond formation involving NH group increases its bending frequency. These results and considerations lead to the conclusion that both C=O and NH sites are implicated in the hydrogen bonds formation.

Due to the almost saturated conditions, the region above 3100 cm\(^{-1}\) was difficult to analyze and no results were carried out.
3.10. BRILLOUIN SPECTROSCOPY

To probe kinetic processes reported to both atropisomerism and self association in concentrated solution of Iopamidol and Iomeprol, Brillouin light scattering technique, in the visible range, was used as a qualitative investigation. BLS VV polarisation spectra were analysed in terms of both position and half-width at half of the maximum of the Brillouin peaks. The experiments were performed with temperature.

First approach was a fast thermal cycle (heating from 25 °C to 85 °C and cooling from 85 °C to 25 °C) to probe changes with temperature on both Iopamidol and Iomeprol concentrated solution in terms of frequency and half-width at half of the maximum of the Brillouin peaks. The second experiment was a kinetics study on the two NICM.

3.10.1. BLS spectra

BLS VV polarization spectra of Iopamidol (Figure 3.29) in the visible range were analysed as direct examination of the quality of the data. The excellent quality, achieved with a high resolution experimental setup can be witnessed. From the raw spectra the standard evolution of the Brillouin shift with temperature can be appreciated, and the figure 3.29 also shows an evident relaxation process.

The spectra show that no changes were detected comparing the initial and the final spectra. Classical DHO analysis for these measurements was used to calculate the frequency ($\nu_{Br}$) and the Half-Width at Half of the Maximum (HWMH; $\Gamma_{Br}$) of Brillouin peaks. In these experiments this data elaboration was useful to plot $\nu_{Br}$ and $\Gamma_{Br}$ as a function of both time (at fixed temperature) and temperature. (figure 3.30). The graphs show that there are no changes in both $\nu_{Br}$ and $\Gamma_{Br}$ parameters.

Fast thermal cycle

In these experiments the DHO analysis was useful to plot $\nu_{Br}$ and $\Gamma_{Br}$ as a function of both time (at fixed temperature) and temperature. (figure 3.30). The graphs show that there are no changes in both $\nu_{Br}$ and $\Gamma_{Br}$ initial and final parameters.
Results and Discussion

Figure 3.29: (left) Overlapping Brillouin spectra during a fast heating and cooling cycle on Iopamidol concentrated solution. (right) Overlapping spectra during the kinetics study on Iopamidol solution

Figure 3.30: (left) Brillouin shift for VIS data of Iopamidol concentrated solution as a function of temperature, from the DHO model. (right) HWMH of Brillouin peaks of Iopamidol concentrated solution as a function of temperature, from the DHO model.

Iopamidol and Iomeprol concentrated solutions were analyzed by a fast thermal cycle. The measures started from 25 °C to 85 °C and again to 25 °C. Three spectra for each NICM were recorded: one at 25 °C before heating, at 85 °C after twenty minutes to allow the thermalization of the sample and the last at 25 °C after cooling. The $\nu_{Br}$ and $\Gamma_{Br}$ of the three spectra were compared to probe kinetics processes.
The same approach was enforced on Iomeprol concentrated solution. Also here the overlapping spectra does not show differences in terms of both shift and width of Brillouin peaks (Figure 3.31).

![Image](image-url)

**Figure 3.31**: (left) Brillouin shift for VIS data of Iomeprol concentrated solution as a function of temperature, from the DHO model. (right) HWMH of Brillouin peaks of Iomeprol concentrated solution as a function of temperature, from the DHO model.

The fast thermal cycle on the two systems does not influence the physical-chemical properties. The concentrated solutions of NICM analyzed do not show an ageing process by this heat treatment.

### 3.10.2. Kinetics studies

To probe the temperature influence on the physical-chemical properties of NICM solutions, another experiment was performed at fixed temperature as a function of the time to investigate kinetics processes in solution.

Iomeprol and Iopamidol concentrated solution were thermalized at 65 °C and 80 °C respectively. These temperatures were maintained 9 hours for Iomeprol (Figure 3.32) and 47 hours for Iopamidol (Figure 3.33). During these time Brillouin spectra were recorded and, from the DHO model, $\nu_{Br}$ and $\Gamma_{Br}$ data of Brillouin peaks were carried out for both the compounds.

From these results the absence of kinetics processes until 85 °C is confirmed. The value fluctuations are smaller than instrumental error (order of ‰).
Figure 3.32: Kinetics study of Iomeprol at 65 °C. (left) Brillouin shift for VIS data of Iomeprol concentrated solution as a function of time, from the DHO model. (right) HWMH for VIS data of Iomeprol concentrated solution as a function of time, from the DHO model.

Figure 3.33: Kinetics study of Iopamidol at 85 °C. (left) Brillouin shift for VIS data of Iopamidol concentrated solution as a function of time, from the DHO model. (right) HWMH for VIS data of Iopamidol concentrated solution as a function of time, from the DHO model.
3.11. CRYSTAL FORMS BY X-RAY DIFFRACTION

The crystal structure of Iomeprol reveals the presence of a molecule in the asymmetric unit of the monoclinic unit cell, space group $P 2_1/n$ (Figure 3.34).

In the refined structure, the iodine atoms are almost coplanar with the phenyl ring, with distances of $0.06\pm0.01\text{Å}$, $0.13\pm0.01\text{Å}$ and $0.16\pm0.01\text{Å}$, respectively, from the mean plane of the carbon atoms. The carboanilido groups are oppositely oriented each-other in the so-called anti-conformation. The substituents of the two amidic functions are oriented on opposite sides with respect to the aromatic ring, with dihedral angles $C_{\text{aromatic}}\text{-}C_{\text{aromatic}}\text{-}C(=O)\text{-}N$ of $88\pm1^\circ$ and $86\pm1^\circ$, respectively. As far as the third phenyl ring substituent is concerned, the dihedral angle formed by the 2-hydroxy-N-methylacetamido group with the aromatic ring (angle: $C_{\text{aromatic}}\text{-}C_{\text{aromatic}}\text{-}N\text{-}C(=O)$) measures $87\pm1^\circ$. As a result of the stereocenters at C9 and C9’, the two hydroxyl groups were found disordered in two different positions. The occupancy factors were refined to a total occupancy value of 1, for each of the two couples of disordered atoms.
Results and Discussion

giving occupancy values of 0.73 and 0.27 for the first disorder group and of 0.69 and 0.31 for the second disordered group.

As expected, the interatomic distances of aromatic ring atoms and their nearest substituents are conservatives with respect to the similar distances found for anhydrous iopamidol, thus the core of NICM maintains its geometry within the different molecules. Overlapping of the residues of iopamidol and iomeprol, both taken from the anhydrous crystals, is shown in Figure 3.35.

![Figure 3.35. Overlapping of the atoms of the core of iomeprol and iopamidol](image)

Main differences between these two molecules are therefore allocated in the side arm structure and conformation which may easily rotate to form hydrogen bonds from the oxygen of the aromatic carbonyl and the amido (O⋯HN) of another molecule and to the alcoholic (O⋯HO) group of a second neighbor molecule.

Thus, the intermolecular network is made by H-bonds / polar interactions between adjacent molecules; long arms of the central iomeprol(1) are connected to one long arm of iomeprol(2) and to the short arm of iomeprol(3), whereas the short arm of iomeprol(1) is connected to a long arm of iomeprol(4). The view of
the elementary cell in a fragment of iomeprol in the three axis direction is provided in Figure 3.36, showing good intermolecular packing, as also demonstrated by the void volume calculation of 2.4%, defined as volume fraction accessible to a water molecule.

![Image](image.png)

**Figure 3.36**: Representation of the unit cell showing two iomeprol pairing

This is probably due to the alternate layers of partially stacked molecules. The layered iomeprol molecules are located in an antiparallel fashion with two pairs of iodine atoms at a distance of 4.25 Å while the two opposite iodine atoms stay at a distance of 6.25 Å.

As far as the molecular conformation is concerned (Figure 3.37), the acetoamido group is in *exo* conformation, according to definitions for atropisomerism, while the two carboanilido groups are in the opposite sides (anti), therefore the conformation of the whole molecule is “exo-anti”[1]
Results and Discussion

Figure 3.37: two atropisomers due to endo-exo and syn-anti equilibria.

A preferred population of the exo conformation in aqueous solution (ca 75%) with respect to the endo one (ca 25%) has been found by NMR data. Within the exo conformation of the side arm, the syn and anti conformations of the two symmetric arms are weighed in favor of the anti one (about 66%). Interestingly enough, the side arms of iopamidol in the crystalline anhydrous form are oriented in the syn-endo conformation (Figure 3.37), while in solution the anti conformation is preferred.

It should be concluded that the conformation preference in the crystalline form is dictated by a more favourable packing and symmetry.

Once the molecular conformation of iomeprol in the crystalline phase is assessed as that also favorably occurring in solution, then solubility of the atropisomer exo-anti could be in principle determined by accurate equilibration of the crystals in solution at constant temperature, by avoiding conformational transitions. Infact, it turns out that the actual commercial formulations of iomeprol (as well as the other NICMs) is always a mixture of the several atropisomers and
that the apparent concentration of the solution seems to be beyond the solubility of the crystalline forms. Still the stability of the solution composition should mainly derive from the high energetic barriers, although the solution composition implies that these energetic differences between the several forms can be somehow overcame. Indeed, several experiments by differential scanning calorimetry on the crystalline powder confirmed by Raman spectroscopic data reveal a sharp solid-solid transition around 120 °C [manuscript in preparation]. Research is in progress to investigate the structure of this polymorph and whether this polymorphic transition is related to a change of packing symmetry alone or to changes in the molecular conformation.

3.12. DSC ON CRYSTAL AND GLASSY STATE

The thermograms of amorphous Iopamidol and Iomeprrol are shown in figure 3.38. The physical aging is higher in Iopamidol.

![Thermograms of Iopamidol and Iomeprrol](image)

**Figure 3.38:** thermograms of Iopamidol and Iomeprrol after physical aging at 140 °C

Figure 3.39 shows the thermograms of amorphous Iopamidol and Iomeprrol. The thermograms carried out by high cooling rate show a small
enthalpy relaxation peak than those carried out by lower cooling speed that are characterized by a large aging.

![Thermograms of Iopamidol and Iomeprol amorphous after an aging cooling process.](image)

**Figura 3.39:** Thermograms of Iopamidol and Iomeprol amorphous after an aging cooling process.

The physical aging can be observed in both thermograms and confirms the glass transition. A comparison of Iopamidol and Iomeprol amorphous was realized. The glass transition, determined by calorimetric measurements, is characterized by the parameters of “onset temperature” ($T_{on}$) and “mid-point temperature” ($T_{mp}$). The onset temperature is determined by the intersection of the tangent to the inflection point of the glass transition curve and the line relating to specific heat of the glass (figure 3.40).
The temperature of the mid-point is the temperature corresponding to the glass transition of 50% of the system. These parameters can be identified in absolute terms only when the glass is not aged.

The enthalpy relaxation generates a sigmoidal curve whose pattern is distorted. To consider the system as a reversible and equivalent thermodynamic process, the "fictive temperature" must be introduced. Even in the presence of enthalpy relaxation, each real curve can be related to a gap of specific heat at $T_{\text{fictive}}$. The definition of $T_f$ implies that the integrals of both $C_p$ relative to the real curve and $C_p$ relative to the curve defined by the gap in temperature $T_f$ must be equal.

To differentiate the behavior of supercooled liquids close to the glass transition, concepts of fragility of glass and the fragility parameter "m" were introduced. Where "m" is defined as a change in a relaxation property as a function of temperature $T > T_g$.

The analytical equation that defines the fragility parameter $m$, relates the logarithm of relaxation time $\tau$ with the ratio of $T_g$ and a temperature $T$ tending to $T_g$. The fragility parameter can be determined on a thermodynamic time scale, conventionally of the order of 100 seconds. These consideration allows to define
the fragility parameter “$m_h$” that can be calculated by correlating the cooling rate and the fictive temperature (equations 3.17) carried out by DSC calorimetry.

$$\frac{d \ln \left( \frac{Q}{1 - \frac{T_{\text{fict}}} {T_f}} \right)} {d \left( \frac{1} {T_{\text{fict}}} \right)} = - \frac{\Delta H^0} {R}$$

$$m_h = \frac{\Delta H^0} {2.303RT_g} \quad 3.17$$

where $Q$ is the cooling rate, $\Delta H^0$ is the activation energy used in various models to simulate the relaxation enthalpy and $R$ is the constant of perfect gases.

From each thermogram, recorded at different scan rate, $T_{\text{fictive}}$ and $T_{\text{onset}}$ were carried out and used to compare the two amorphous (Iopamidol and Iomeprol). The following table (table 3.9) shows the values of $T_{\text{fictive}}$ and $T_{\text{onset}}$ at different scan rate for both Iopamidol and Iomeprol.

**Table 3.9**: Fictive and onset temperatures of Iopemidol and Iomeprol amorphous.

<table>
<thead>
<tr>
<th>Scan Rate</th>
<th>Iopamidol</th>
<th>Iomeprol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_{\text{fictive}}$</td>
<td>$T_{\text{onset}}$</td>
</tr>
<tr>
<td>20</td>
<td>152</td>
<td>153</td>
</tr>
<tr>
<td>10</td>
<td>151</td>
<td>154</td>
</tr>
<tr>
<td>5</td>
<td>148</td>
<td>155</td>
</tr>
<tr>
<td>2</td>
<td>146</td>
<td>156</td>
</tr>
<tr>
<td>1</td>
<td>143</td>
<td>158</td>
</tr>
<tr>
<td>0.5</td>
<td>140</td>
<td>159</td>
</tr>
</tbody>
</table>

The data of $T_{\text{fictive}}$ and $T_{\text{onset}}$ at different cooling rates are shown in the figure 3.41. The $T_{\text{onset}}$ increases with decreasing cooling rate, while the $T_{\text{fictive}}$ shows an opposite behaviour. By plotting the logarithm of cooling rate versus the inverse of $T_{\text{fictive}} m_h$ was calculated (figure 3.42).
Results and Discussion

Figure 3.41. Trends of $T_{\text{fictive}}$ and $T_{\text{onset}}$ of both Iopamidol and Iomeprol at different cooling rates

Figure 3.42. Graphs of logarithm of cooling rate vs inverse $T_{\text{fictive}}$

The slope of the two lines passing through the experimental points indicates the activation enthalpy that is related to the value of $m_h$ for supercooled liquids. By using the equations described above the values of $m_h$ were calculated for both Iopamidol and Iomeprol.
Results and Discussion

3.18
For Iomeprol more interesting results were carried out (Figure 3.43). These results show the presence of a reversible solid-solid phase transition similar to that found for the ammonium nitrate$^{[19]}$. The Iomeprol powder is micro-crystalline (supported by a series of experiments using other techniques) and its solid-solid transition occurs into the range of glass transition. The solid-solid transition could involving an hydrogen bond because the enthalpy value of the process is about 9400 J/mol. For Iopamidol no significant results were revealed after aging process (figure 3.43).

\[
\frac{d \ln |Q|}{d \left( \frac{1}{T_{foz}} \right)} = -\frac{\Delta H^0}{R} = -61107,
\]

\[
m_{i} = \frac{\Delta H^0}{2.303RT_{foz}} = \frac{61107}{2.303(146 + 273)} \approx 60
\]

3.19
\[
\frac{d \ln |Q|}{d \left( \frac{1}{T_{foz}} \right)} = -\frac{\Delta H^0}{R} = -52751,
\]

\[
m_{i} = \frac{\Delta H^0}{2.303RT_{foz}} = \frac{52751}{2.303(152 + 273)} \approx 50
\]

For Iomeprol more interesting results were carried out (Figure 3.43). These results show the presence of a reversible solid-solid phase transition similar to that found for the ammonium nitrate$^{[19]}$. The Iomeprol powder is micro-crystalline (supported by a series of experiments using other techniques) and its solid-solid transition occurs into the range of glass transition. The solid-solid transition could involving an hydrogen bond because the enthalpy value of the process is about 9400 J/mol. For Iopamidol no significant results were revealed after aging process (figure 3.43).

Figure 3.43: cooling physical aging for both Iomeprol and Iopamidol powder
To study the solid-solid transition of Iomeprol powder, a new series of aging calorimetric experiments was performed. Different heating rates were used (20, 10, 5, 2, 1 and 0.5 °C/min), while the cooling rate was constant for each measure and its value was 20 °C/min starting from 200 °C to 80 °C (figure 3.44).

![Figure 3.44. calorimetric measures by different heating rate of Iomeprol powder.](image)

### 3.13. MICRO-RAMAN SPECTROSCOPY

To probe the solid-solid transition in both powder and glassy Iomeprol, MicroRaman measurements were carried out at room temperature and by temperature gradient.

The data show:
1. The transition is observed between 105 °C and 115 °C (figure 3.45).
2. After the transition the crystalline form is similar to the glass form. The comparison of the two Raman spectra confirms this fact (figure 3.46).
Results and Discussion

Figure 3.45: transition of Iomeprol powder. Raman micro-spectroscopy spectral subtractions graph as a function of temperature.

Figure 3.46: Spectra of Iomeprol before (start) and after (end) the transition. (top) Overlapping spectra of both crystalline Iomeprol after the transition and glass Iomeprol.
The Iopamidol powder, the amorphous solids of both Iopamidol and Iomeprol (obtained by heat treatment at 110 °C for 17 hours into DSC 6 calorimeter), the Iopamidol pentahydrate solid (as precipitate into the commercial vials) were analyzed at room temperature to carry out more possible information on these systems. These measurements allowed to get both a spectra database of these five systems (Figure 3.47) and their comparison.

**Figure 3.47.** (right) Raman spectra at room temperature of Iomeprol glass and Iomeprol crystal. (left) Raman spectra at room temperature of Iopamidol glass, pentahydrate Iopamidol and Iopamidol crystalline powder.

Comparing Iomeprol crystal and Iomeprol galss spectra, changes in peak bands are visible at 200-400 cm\(^{-1}\), 700-900 cm\(^{-1}\) and 1600-1700 cm\(^{-1}\) (Figure 3.48). Furthermore, a general broadening of the bands in the Iomeprol galss spectrum is verified, while Iomeprol crystal shows sharp bands.

Figure 3.49 shows the experimental Raman spectra of both amorphous and crystalline Iomeprol in comparison with Raman spectrum calculated by the simulations.
Results and Discussion

Figure 3.48. Comparison of Iomeprol both amorphous and cristalline.

Figure 3.49: Comparison between experimental spectra of amorphous, cristalline Iomeprol and Iomeprol spectrum calculated.
Raman microscopy measures as a function of temperature were very interesting to study the solid-solid transition observed in the Iomeprol powder. The same study was performed on Iopamidol pentahydrate crystal.

The graph of the spectral differences as a function of temperature (Figure 3.50) shows the presence of changes in the bands of the spectra. In particular, three slope changes in the curve (three transitions) that can be compared with the three thermal phenomena (peaks overlapping) that are observed in the calorimetric thermogram, recorded by calorimeter Perkin Elmer DSC 6 using the same temperature range and scan rate (Figure 3.51).

![Graph showing spectral differences as a function of temperature for Iopamidol crystalline 5H2O and glass](image)

**Figure 3.50:** Graph of the spectral differences as a function of temperature for Iopamidol pentahydrate and for Iopamidol glass.

Also in this case, as for Iomeprol (figure 3.46), a similarity can be observed between the spectra of Iopamidol after the transition and amorphous Iopamidol (figure 3.52). From this image the differences between initial (before transition) and final (after transition) spectra is shown. An enlargement of the bands was observed.
**Figure 3.51.** Thermogram of Iopamidol pentahydrate between 25 °C and 120 °C, scan rate was 2 °C/min.

**Figure 3.52.** Comparison of initial spectrum (start) before the transition, and the final spectrum (end) after the transition of pentahydrate Iopamidol. Overlapping of final and amorphous Iopamidol spectra was done.
Additional Raman measures were performed in the range temperature containing the glassy transition, but no changes in the spectra were observed for both Iopamidol and Iomeprol.
3.14. BIBLIOGRAPHY


CONCLUSIONS

Interactions are the basis of a good efficiency of NICMs because they promote the association phenomenon that leads to a decrease of the number of particles in solution, thus the osmolality. In these systems, the intermolecular interactions evolve over time because they are governed by the aggregation equilibrium that involve a moderate number of molecules of NICMs. Thus, the intermolecular interactions play a very important role in terms of both efficiency and stability of the concentrated solutions. Calorimetric measurements, performed as a function of concentration, allowed to evaluate and to quantify the heats enveloped or absorbed during a dilution process whose values give mainly information about the nature of the interactions between NICM-H$_2$O. By elaborating osmolality data from literature as a function of concentration, interactions NICM-NICM were probed and their presence were confirmed by the ideality deviation of osmotic coefficient. The NICMs can assume various molecular conformations due to the atropisomerism. The possibility of the NICM molecules to assume various molecular conformations could promote several types of association in which there is not an effective stacking (nucleation), but a “disordered” aggregate characterized by its own aggregation constant.

The amount of the data obtained give a number of elements to formulate an aggregation mechanism. Precipitation precesses were made to determine which atropisomers and interactions are involved into the elementary crystal cell. Experiments as a function of temperature highlighted a complexity into the solid of NICMs in terms of physico-chemical properties because a polymorphic transition was found as a function of temperature. A detailed study at molecular level, in terms of side chains, could give information on the contibutes of each moiety to the intermolecular interactions. This would mean to modulate the association process an so the physico-chemical properties of NICMs.
PAPERS
Interactions in iodinated contrast media solutions

Part 1. A thermodynamic study

G. Giannini · F. Cuppo · L. Fontanive · N. D’Amelio · A. Cesàro · A. Maiocchi · F. Uggeri

Abstract Contrast media are synthetic molecules often characterized by the presence of heavy atoms, such as iodine, widely used in diagnostic studies. In the framework of a study on the physico-chemical of non-ionic contrast media (NICM), this study reports the calorimetric data for a characterization of the thermodynamic behavior of the aqueous solutions of three NICMs, namely, iopamidol, iomeprol, and iopromide, characterized by the presence of three iodine atoms in the benzene ring. Hydrophilicity is provided by three side arms with polar groups. Here, the results of a calorimetric investigation on the heat of dilution of iopamidol, iomeprol, and iopromide and on iomeprol–iopamidol mixture are illustrated. Despite the very similar chemical structures, the dilution process of iopamidol and iopromide was found exothermic, while an endothermic dilution is shown by iomeprol. The results are discussed in terms of the few other literature data and on the basis of structural and conformational properties.

Keywords Iodinated contrast media · Iopamidol · Iomeprol · Heat of dilution · Solution thermodynamics

Introduction

Non-ionic contrast media (NICM) are nowadays considered mature investigative drugs of great relevance in delicate radiological analysis like angiography, urography, and myelography [1]. The generic structure of NICM is characterized by a 2, 4, 6 tri-iodo benzene substituted in 1, 3, 5 with non-ionic highly hydrophilic arms containing both amido linkages and hydroxyl substituents [1]. The success of these molecules mainly resides in some peculiar physico-chemical properties that associate relatively low-osmolality with relatively low-viscosity up to high concentration in water [2–4], as well as in the stability of the aromatic iodine substitution and the high concentration of iodine per molecule (up about 50% in weight). The side chains are significantly important not only for increasing the aqueous solubility, but also because they decrease the toxicity and raise the elimination of substances from the patient body [3]. Several investigations have been carried out in the recent times to introduce variations in the structure of the side arms and to improve the solubility without decreasing the iodine weight content [1, 5].

Iopamidol, iomeprol, and iopromide are injectable iodinated contrast agents, and they belong to the class of non-ionic contrast media [6]. The molecular structure of these compounds is given in Fig. 1.

Despite the large use in the clinical practice and the great interest from the molecular viewpoint of these molecules, literature studies on the physico-chemical properties are scarce and almost limited to some properties related to the debate about the association equilibria [7–10] and to phenomenon of atropisomerism characteristic of the crowded substitution on the aromatic ring [1, 11]. As a consequence, the understanding of the correlations between
molecular structure and peculiar physico-chemical properties does not appear satisfactory.

A study aimed at characterizing the properties of some NICMs in solution and in the solid state by means of thermodynamic and spectroscopic methods has been carried out. The solution thermodynamics investigation concerns the correlation of the osmolality (free-energy) with calorimetric (enthalpy) and density (volume) properties of the solutions as a function of concentration. Since many molecular descriptors of the solution properties of these molecules are conformation-dependent and, therefore, complicated by the existence of metastable conformers population, a detailed $^{13}$C-NMR study on iopamidol, iomeprol, and iopromide and on common simplified primers has also been carried out in parallel in order to assigning the most relevant conformers (L. Fontanive, unpublished results). This preliminary valuable information is useful for the discussion of the macroscopic effects of the concentrated solution of NICMs.

Although calorimetry has been used even recently for studying subtle interactions occurring in aqueous solutions of drugs [12] and other pharmaceutical excipients, including the elucidation of polymorphism [13], to the best of our knowledge, contrast media have not been investigated yet. Here, a report is presented about the heats of dilution of the solutions of the three NICMs, iopamidol, iomeprol, and iopromide, in the concentration range up to approximately 1.6 m at 25 and 37 °C. These data help to understand some subtle differences in the solution behavior of the three different compounds, which is not fully evident at first glance from other experimental results.

**Fig. 1** Chemical structures of iopamidol (a), iomeprol (b), and iopromide (c). Note the structural differences in the three hydrophylic side arms

**Experimental**

**Materials**

Iopamidol, iomeprol, and iopromide solutions are commercially available as aqueous solutions at molal concentration ($m$) of 1.48, 1.67, and 1.52 m, for iopamidol, iomeprol, and iopromide, respectively, and were used for the calorimetric measurements without any further purification. In addition to commercial solutions, an aqueous solution of iopamidol was also prepared from solid powder at the same concentration of vials. The solution was prepared by dissolving the iopamidol powder by stirring the weighed solid in a weighed amount of water at 65 °C for 30 min and then heating up to 85 °C for 10 min. These measurements were used as a control for assessing any possible influence on the thermal properties due to the trace presence of excipients in the commercial solutions.

**Methods**

Heats of dilution measurements on NICM solutions were carried with an LKB 10700 batch-type microcalorimeter equipped with gold cells and were performed at two temperatures, 25 and 37 °C. The electric circuit of the control unit of the calorimeter was modified to allow it to operate up to 45 °C. Electrical calibration of the heat effects was performed and averaged over many independent data points, to give a calibration constant $K = 1.77 \times 10^{-5}$ J/W. The temperature, once set, was checked directly inside the thermostatted air bath. The output signal of the thermopiles, amplified by a Keithley 150B microammeter, was connected to a PC through a Picolog® A/D data acquisition interface. The capability of the digital acquisition was limited to 1 datapoint/s as an average over 10 points. Mathematical analysis of the calorimeter data (baseline correction, integral) was carried out using the Microcal ORIGIN® graphic package.

In each measurement a weighed amount of solution (2 mL) at initial molal concentration $m_i$ was mixed in the sample cell with a weighed amount of water (2 or 1 mL) to give a solution of known final concentration $m_f$. Dilution series were carried out starting from the commercially available aqueous solutions and were continued, at each step diluting the previously obtained sample, until the limit of experimental accuracy was reached, in order to obtain the most accurate evaluation at infinite dilution limit. Given the viscosity of the samples, in particular of the most concentrated ones, a single mixing step of rotating drum
containing the cells was not sufficient to obtain a complete dilution of the sample, as revealed by a significant heat effect still observed by performing a second rotation. Therefore, subsequent rotational movements (up to 15, for the most concentrated samples) of the calorimetric unit (at time intervals of about 2 min) were performed until no residual heat effect was observed. Figure 2 shows the calorimetric curves of an endothermic heat dilution series, overlapped for comparison purpose. In the figure, bumps corresponding to rotations of calorimetric cell are well visible especially for the curves of systems at high concentration, with changes in the shape and the area of the curves upon dilution.

Results and discussion

Isothermal calorimetric dilution measurements on NICM solutions were collected as described in the Experimental part and for each experiment the heat of dilution per mole of solute was calculated for the dilution step from \( m_i \) to \( m_f \). Table 1 reports all the relevant experimental data for the systems and the conditions investigated. Iopamidol solution from commercial vials at initial molal concentration of 1.48 was subjected to subsequent dilutions performed at 25 and 37 °C, providing a thermal response from +1.73 to about 0.02 J. Similarly, heat of dilution data of iomeprol solution (initial concentration \( m = 1.67 \)) and iopromide solution (initial concentration \( m = 1.52 \)) as a function of concentration have been collected at 25 and 37 °C in the same experimental conditions of iopamidol. Table 1 reports also the data of the dilution experiments for solutions freshly prepared by dissolving the anhydrous iopamidol powder. It is clear that all the experimental heats of dilution data are constantly exothermic for iopamidol and iopromide solutions and endothermic for iomeprol solutions.

Given the method of subsequent dilution, treatment of the raw calorimetric data has been simply carried out by

<table>
<thead>
<tr>
<th>Sample</th>
<th>( m_i )</th>
<th>( m_f )</th>
<th>( Q_{dil}/J )</th>
<th>( \Delta H_{dil}/J ) mol(^{-1} )</th>
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<td>-725</td>
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<td>+0.593</td>
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<td>+0.258</td>
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<td>0.051</td>
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<td>0.165</td>
<td>0.080</td>
<td>-0.082</td>
<td>+261</td>
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<td>0.073</td>
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<td>-53.7</td>
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</table>

\( ^a \) This series of experiments refers to the solution of iopamidol freshly prepared from the anhydrous semicrystalline powder

Fig. 2 Calorimetric curves of heat of dilution series at 37 °C (iomeprol); the original curves are overimposed starting at the same initial time of the mixing process. Initial concentration of iomeprol solution decreases from top to bottom curve
summing up the heat of dilution for each dilution step to plot the heat of dilution as a function of the final concentration ($m_f$). Extrapolation of the heat of dilution of the solution to concentration $m_f = 0$ has been done according to standard methods of fitting [14].

Before the analysis of the data, let us comment on the reproducibility of experimental results and of the comparison between the data at 25 °C with the only literature report on these systems. Figure 3 reports two set of data at 37 °C; one experiment is carried out by using the solution of the commercial vials at initial concentration 1.48 m, the other carried out on a solution at same concentration freshly prepared from iopamidol semicrystalline powder by warm dissolution as described in the experimental section. The experiments performed on iopamidol solution directly prepared from powder do not show detectable differences with commercial vials. These results also imply that sample history effects due to atropisomeric non-equilibrium phenomena are excluded or are thermally insignificant.

From the polynomial fitting of the curves of the heat of dilution of the three systems, the apparent molar relative enthalpy, $L\phi$, was calculated as a function of $m$:

$$L\phi = \frac{L - L^0}{m^2} = \frac{L}{m^2}$$

$$\Delta H_{dil} = H_{final} - H_{initial} = L\phi(m) - L\phi(m_i)$$

According to the formalism introduced by Friedman [15] and thereafter extended by Kauzmann [16] to non-electrolyte solution, the molar apparent relative enthalpy is evaluated from a series of consecutive dilutions to approximately infinite dilution (or extrapolated to this limit) where $m_f = 0$ in the Eq. 2 and then [17]:

$$-\Delta H_{dil} = L\phi(m) = h_{ii}m + 2h_{ii}m^2 + \ldots$$

The values of the coefficients for the three systems are:

$L\phi$ (Iopamidol): $2771m - 1054m^2 + 255m^3$ (at 25 °C)

1986$m - 570m^2 - 114m^3$ (at 37 °C)

$L\phi$ (Iomeprol): $-2947m + 2898m^2 - 1743m^3 + 413m^4$

(at 25 °C)

$-3798m + 3523m^2 - 1898m^3 + 401m^4$

(at 37 °C)

$L\phi$ (Iopromide): $2287m - 948m^2 + 242m^3$ (at 25 °C)

$876m - 47m^2$ (at 37 °C)

Figure 4 shows the fitted curves of the apparent molar relative enthalpy of the solution, together with the experimental data points associated to each curve (the experimental values have been subtracted by the values extrapolated at $m = 0$).

The most important result lies in the evidence that dilution of iopamidol and iopromide is an exothermic process, whereas for iomeprol, dilution is endothermic. Such a neat difference must certainly be explained in terms of substantially different balance of the solutions energetics, where both solute–solvent and solute–solvent interactions are involved. The rank of the heat of dilution agrees with the order found in the reorientation dynamics of iopromide and iopamidol as measured by NMR as well as in the water/butanol partition coefficient [18]. From the calorimetric data it also appears that the behavior of iopromide dilution at 25 °C is similar to the behavior of iopamidol at 37 °C. As far as the absolute values of heat of dilution are concerned, these are relatively small; in fact,
intermediate heat of dilution does not exceed about 2–3 kJ mol\(^{-1}\) for the complete dilution process, starting from concentrated solutions and down to infinite dilution limit. The absolute value of molar apparent relative enthalpy for iomeprol is lower than for iopamidol and iopromide in all range of concentrations. Data for iopamidol at 25 °C were also compared with the literature data at 25 °C [19], showing only a small difference in the integral heats of dilution at low concentration. These literature data where not further processed as they were used to correct the heat of mixing with proteins (fibrinogen or lysozyme). It has been pointed out that a small endothermic effect was measured and ascribed to changes in the solvation properties of the proteins and not to direct interactions, as confirmed by the absence of spectroscopic Raman evidence [19].

The values of the heat of dilution decrease with temperature for all three NICMs, as it can be seen from two sets of experiments at 25 and 37 °C. From these data an evaluation of the heat capacity terms can be approximately carried out. From the value of Fig. 4 the heat capacity of iopromide solution results almost twice of that of iomeprol and iopamidol. This difference is almost surprising in view of the similar chemical structure of the NICMs and should be ascribed to the greater increase of degree of freedom of the solvated species of iopromide respect to iopamidol and iomeprol. Whether the \(C_p\) changes are also related to the variation in the potential associative equilibria in solution can only be ascertained by independent measurements. Furthermore, direct \(C_p\) experiments need to be carried out.

Before commenting the solution properties of NICMs on the basis of the other literature data, let us report about the calorimetric heat of dilution experiments carried out at 25 °C on mixtures of iomeprol with iopamidol, with the purpose of detecting the change in the heterotactic interactions (if any) of these two similar systems, but with opposite sign of the enthalpic term upon dilution. Measurements were made on mixtures at three solute concentration ratios, in order to evaluate the compatibility of the solutes in aqueous solutions. The results of these experiments are summarized in Table 2.

Figure 5 shows heat of dilution data plotted against the concentration of iomeprol and reports data of iopamidol and iomeprol at 25 °C already calculated. The linearity of plot of Fig. 5 suggests that the excess heat of mixing (\(h_{ij}\) in the notation of Eq. 3) of iopamidol and iomeprol is almost zero and that their mixed interactions are enthalpically equivalent. The heat of dilution of a 60% iomeprol mixture would be zero, and therefore, such a mixed system is athermic with contributions to the mixing of entropic nature only.

That both intermolecular association and hydration phenomena govern the solution properties of NICMs is shown by many literature evidences. The decrease of the osmotic coefficient for iopamidol and iomeprol [10, 20] is typical of a system undergoing a progressive association, although its analysis is hampered by the clear evidence of strong hydration, which alter the most straightforward analysis commonly used for these processes [21–23]. The extensive hydration of NICMs can be easily deduced from a qualitative analysis of the hydrodynamic volumes evaluated by viscosity measurements. Obviously, both solute–solute interactions and solvent–solute interactions must contribute to the observed thermal effects; the data here reported clearly show that a prevalence of hydrophilic interactions occurs in the solution of iomeprol with respect to the prevalence of less polar interactions occurring in iopamidol and iopromide solution.

In addition, possible concentration-dependent and temperature-dependent conformational effects should also be taken into account. Indeed, the conformational properties of NICMs in solution are dominated by a large number of potential conformers, some of which fall in the category of atropisomerism [1]. Thus, many molecular descriptors of the solution properties of these molecules are conformation-dependent and, therefore, complicated by the existence of metastable conformers population. A detailed \(^{13}\)C-NMR study on iopamidol, iomeprol, and iopromide and on common simplified primers is in progress for the assignment of most relevant conformers in NICMs.

![Fig. 5 Heat of dilutions of iomeprol/iopamidol mixture at 25 °C](image)
(L. Fontanive, unpublished results). Without going into the detail of the conformational variability and of the $^{13}$C-NMR $^1$H-NMR investigations carried out for the elucidation of the most probable conformers, the important result is that only six conformers seem to be predominant for iomeprol, while only two for iopamidol. Although these results derive from the parallel study to be published (L. Fontanive, unpublished results), let us capitalize this information in view of the fact that no changes in the population were observed as long as the temperature was changed from 25 to 37°C. Some preliminary information about the interactions is also available and can be useful for the discussion of the macroscopic effects of the concentrated solution of NICMs. Among the several potential isomers, only intermolecular interactions between polar hydrophilic groups have been detected. Therefore, other non-polar interactions must be detected as inferred by the calorimetric results here presented that allow rationalizing the profile of energetics of the interaction in NICM solutions.

In conclusion, the analysis of the thermodynamic properties of NICM can be fruitfully accomplished in terms of balance between polar and non-polar homotactic interactions and of different contributions from the hydration water. Spectroscopic data are necessary to attribute to specific functional groups the role on interacting moieties responsible for the weak aggregation and, therefore, to both low-viscosity and low-osmolality behavior in solution.

Acknowledgements Research carried in the frame of a collaboration of Department of Life Sciences with Bracco Research Center under the Project “Physical Chemistry of Iodinated Contrast Media”.

References

NMR Reinvestigation of the Caffeine–Chlorogenate Complex in Aqueous Solution and in Coffee Brews

Nicola D’Amelio · Luca Fontanive · Fulvio Uggeri · Furio Suggi-Liverani · Luciano Navarini

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Abstract Caffeine complexation by chlorogenic acid (3-caffeoylquinic acid, CAS Number [327-97-9]) in aqueous solution as well as caffeine–chlorogenate complex in freshly prepared coffee brews have been investigated by high-resolution $^1$H-NMR. Caffeine and chlorogenic acid self-associations have also been studied and self-association constants have been determined resorting to both classical isodesmic model and a recently introduced method of data analysis able to provide also the critical aggregation concentration (cac). Furthermore, caffeine–chlorogenate association constant was measured. For the caffeine, the average value of the self-association constant determined by isodesmic model ($K_a=7.6\pm0.5 \text{ M}^{-1}$) is in good agreement with the average value ($K_a=10\pm1.8 \text{ M}^{-1}$) determined with the method which permits the determination of the cac ($8.43\pm0.05 \text{ mM}$). Chlorogenic acid shows a slight decreased tendency to aggregation with a lower average value of association constants ($K_i=2.8\pm0.6 \text{ M}^{-1}$; $K_a=3.4\pm0.6 \text{ M}^{-1}$) and a critical concentration equal to $24\pm1 \text{ mM}$. The value of the association constant of the caffeine–chlorogenate complex (30±4 M$^{-1}$) is compatible with previous studies and within the typical range of reported association constants for other caffeine–polyphenol complexes. Structural features of the complex have also been investigated, and the complex conformation has been rediscussed. Caffeine chemical shifts comparison (monomeric, complexed, coffee brews) clearly indicates a significant amount of caffeine is complexed in beverage real system, being chlorogenate ions the main complexing agents.

Keywords Caffeine · Chlorogenic acid · NMR · Caffeine–chlorogenate complex · Espresso coffee

Abbreviations
BBI Broadband Inverse
cac Critical aggregation concentration
COSY Correlation spectroscopy
HSQC Heteronuclear single quantum coherence
HMBC Heteronuclear multiple bond correlation
NOE Nuclear overhauser effect
NOESY Nuclear overhauser effect spectroscopy
ROESY Rotating frame overhauser spectroscopy
TOCSY Total correlation spectroscopy

Introduction

It has long been known that caffeine interacts with polyphenolic molecules in aqueous solution.$^1$ Several complexes of caffeine with polyphenols$^2$ and aromatic
hydroxy acids such as methyl gallate, 3-nitrobenzoic acid, 5-chlorosalicylic acid, pyrogallol, catechins, theaflavin, gallic acid and quercitin, and cyclodextrins, have been investigated. The complex formed between caffeine and chlorogenate, a well-known major polyphenolic constituent of green coffee bean, was isolated one century ago by Gorter. Sondheimer et al. determined the equilibrium constant of the complex reaction in water by spectrophotometric method. The same method has been used by Kappeler et al. to study caffeine as well as other purine alkaloids complexation with chlorogenate. In the ‘70s, Horman and Viani, on the basis of nuclear magnetic resonance (NMR) chemical shift data, proposed that the caffeine–chlorogenate complex might be described as a 1:1 hydrophobically bound π-molecular complex. In the same study, the association constant was determined and the portion of the chlorogenate ion involved in the complex formation was identified. In particular, it was proposed that the plane of the caffeine molecule is parallel to the plane of the aromatic ring of the caffeoyl ester group and that the five and six-membered rings of the nitrogen heterocycle are equally involved in complex formation. It has to be stressed out that the same authors reported two slightly different conformations of the caffeine–chlorogenate complex and suggested that the conformation is not absolutely fixed, but represents the time average of many other conformations involving relative twisting, sliding, and rocking of the two components. This model was found strikingly resembling the crystal structure of the caffeine–potassium chlorogenate complex elucidated 15 years later by Martin et al. On the basis of this similarity, it has been suggested that the complexes discussed by Horman and Viani are precursors in crystal formation and growth. In the same work, the role played by the potassium ion was emphasized. It is to be noted that as chlorogenic acid, both 3-caffeoylquinic acid [327-97-9] and 5-caffeoylquinic acid [906-33-2] have been mentioned in the literature; however, it is the former that has been studied in association with caffeine, and it is the only one easily commercially available.

Apart the above-mentioned earliest studies, caffeine–chlorogenate complexation has not been the subject of further NMR studies. The caffeine–chlorogenate complexation has been indicated as crucial mechanism to explain the compartmentation of caffeine in coffee plants and the qualitative relationship between caffeine and chlorogenic acid content distribution among wild Coffea species and, very recently, as a possible strategy to develop an analytical method to determine caffeine by means of capillary electrophoresis.

Caffeine has also been shown to self-associate in aqueous media and several studies reported on proton NMR chemical shift data and self-association molecular models.

Similarly, polyphenolic molecules such as methyl gallate, and theaflavin show self-association in aqueous media. Also in this case, proton NMR proofs to be an important tool to determine association constants and molecular model details.

The caffeine–chlorogenate association constant (16.9 M⁻¹ in D₂O at 40°C) reported in the earliest literature, never reproduced from that time, is confined at the lower side of the range reported for other caffeine–polyphenols complexes (10–200 M⁻¹). The recent advances in NMR spectroscopy equipment and methods make possible nowadays to assess a wider range of solute concentration in aqueous media, to study the self association influence on the complex formation as well as to elucidate structural features of the complex. The self-association of chlorogenic acid, as far as we know, has not yet been subject of detailed investigation.

Herein, the caffeine–chlorogenate complex has also been studied in freshly prepared coffee brews. The application of high-resolution ¹H-NMR spectroscopy to study coffee brews “as prepared” without any pretreatment has been almost unexplored. A part from the earlier studies by Horman and Viani on 15% w/w lyophilized “home brew” infusions in D₂O, only recently, freshly prepared coffee brews, including espresso coffee, have been characterized in detail by Bosco et al. by using H₂O/DMSO-d₆/HCl solvent system and by Tavares and Ferreira under similar conditions but without HCl. In both investigations, however, differently from the earlier studies, the caffeine signals assignment has been reported without mentioning caffeine self-association as well as caffeine complex formation with chlorogenic acid. Similarly, the chlorogenic acid spectral features have been described without mentioning possible self- and/or hetero-associations.

In the present work, espresso coffee brew has been chosen as beverage model system because differently from coffee infusions which are true solutions of coffee solubles, in espresso coffee, the presence of emulsified lipids may represent an additional source of competitive systems for caffeine complexes formation.

Materials and Methods

Caffeine, chlorogenic acid (CAS Number [327-97-9]), and all other chemicals were purchased from Sigma Aldrich (Sigma Chemical Corp., St. Louis, MO, USA) and used without further purification. Solutions containing chlorogenic acids were prepared in 80 mM phosphate buffer at pH 7 in order to prevent pH effect on chemical shift at different concentrations. Caffeine and chlorogenic acid were dissolved in D₂O (99.9% purity) containing sodium 3-(trimethyl-silyl)propionate-2,2,3,3,d₄ (TSP) as internal standard for chemical shift referencing. Coffee samples were added with about 10% D₂O containing TSP.

Medium roasted pure Coffea arabica blend coffee (illycaffè S.p.A., Italy) was used properly ground at an
appropriate particle size distribution by using a professional grinder (Luigi Mazzer s.r.l., Italy). Three different production lots were used. Espresso coffee brew was prepared by using a professional espresso coffee machine (La Marzocco s.r.l., Italy) under the following conditions: coffee powder 13.5 g and extract (beverage) mass 50±5 g (regular espresso double shot) according to espresso coffee preparation standard.29 Four different beverages for each lot have been collected together to get espresso coffee samples (measured pH ranging from 5.1 to 5.13).

The espresso coffee samples prepared under experimental conditions of the present work are characterized by a caffeine content in the range 15–20 mM and chlorogenic acids (cumulative 3-, 4-, and 5-caffeoylquinic acids) close to 10 mM.30

All NMR measurements were performed at 300 K on a Bruker Avance III Ultra Shield Plus 600 MHz spectrometer provided with a two-channel Broadband Inverse probe. Assignment of caffeine, chlorogenic acid, and main components in coffee were accomplished by a series of 2D spectra, namely 2D-nuclear overhauser effect spectroscopy (NOESY), 2D-correlation spectroscopy (COSY), 2D-total correlation spectroscopy (TOCSY), 1H,13C-heteronuclear multiple quantum coherence (HSQC), 2D-1H,1H-TOCSY,1H,13C HSQC, and 2D-1H,13C-heteronuclear multiple bond correlation.

TOCSY spectra were recorded with a total spin-locking time of 75 ms using a MLEV-17 mixing sequence; ROESY spectra were performed with a standard pulse sequence using a ROESY spinlock of 350 ms and a power of 2.3 KHz. During all 2D experiments, water suppression was achieved by excitation sculpting.31 The spectral width of homonuclear 2D experiments was typically 6,600 Hz in both F1 and F2 dimensions.

NOESY spectra were recorded with mixing times ranging from 300 ms to 1.2 s in order to reconstruct nuclear overhauser effect (NOE) buildup curves. Distance constraints were inferred by spectra recorded with a mixing of 300 ms at which spin diffusion phenomena were not present (linear part of NOE buildup). Intermolecular NOEs were converted into distance constraints by imposing a maximum internuclear distance of 0.5 nm. Structure calculation was performed with Hyperchem (release 8.0 for Window, Hypercube Inc., 2007) using molecular mechanics (MM+ method). Energy minimization was achieved by conjugate gradient method.

Numbering of molecules is reported in Figure 4.

Results and Discussion

The interaction between caffeine and chlorogenic acid was characterized by monitoring chemical shift deviations upon addition of chlorogenic acid to a solution of pure caffeine in D2O. Structural features of the complex were inferred by 2D-1H,1H-NOESY spectroscopy.

Since aromatic molecules tend to aggregate in aqueous media, we first measured the association tendency of caffeine and chlorogenic acid, in order to find the optimal concentrations to study the interaction without the interference of their multimeric forms.

Caffeine Self-Association

Caffeine is known to self-associate in aqueous solution with the formation of dimers or higher aggregates by “vertical-stacking” and the thermodynamics of such behavior as well as possible geometries of stacked caffeine molecules have been studied in detail.32-34 Several experimental techniques have been used to determine the caffeine self-association constant in aqueous media35 including NMR. In particular, by measuring chemical shift changes on dilution and by fitting the experimental results with appropriate models, it is possible to accurately obtain the self-association strength as described by an equilibrium constant which has units of inverse molarity. As far as the model is concerned, both the indefinite noncooperative and cooperative association models have been used.36 The indefinite noncooperative association model, well known as isodesmic model,37 assumes that the equilibrium constant for the formation of the An+1 species from A and An is always the same. In other words, the equilibrium constant for the formation of a trimer from a dimer is the same as that describing the formation of a dimer from the free species.

\[
A + A \rightarrow A_2 \quad K \\
A_2 + A \rightarrow A_3 \quad K \\
nA \rightarrow A_n \quad K_1 = K^n
\]  

On the contrary, in the indefinite cooperative model of molecular self-association, the equilibrium constants of reactions (Eq. 1) are assumed to be equal for all n>2 with a value of σK where σ is the cooperativity coefficient. Davies et al.38 determined a cooperativity coefficient equal to 1.08±0.02 confirming the use of the isodesmic model as the more appropriate one in the case of caffeine self-association.

In this study, we used both the isodesmic model and a recently introduced method of data analysis able to provide the critical aggregation concentration (cac) of the associating system in addition to the self-association constant.

In the assumption that the chemical shift of the aggregates does not depend on the number of molecules involved and considering the fast exchange condition on the NMR time scale, the equilibrium constant K of each single step can be derived plotting the observed chemical...
shift of one caffeine signal as a function of the concentration and fitting the obtained curve with the following equation\textsuperscript{22}:

\[ \delta_{\text{obs}} = \delta_m + \Delta_0 K[C][2 - K[C]] \]

\[ [C] = C \left[ \frac{2}{1 + \sqrt{4KC + 1}} \right]^2 \]  

where \( \delta_m \) is the chemical shift of the monomer, \( \Delta_0 \) is the limiting deviation of the aggregate from \( \delta_m = \delta_{\text{aggr}} - \delta_m \), \([C]\) is the equilibrium concentration of the monomer, and \( C \) the global concentration of the species.

We obtained an average value of \( K = 7.6 \pm 0.5 \text{ M}^{-1} \) (Figure 1a) in very good agreement with what reported elsewhere and briefly reviewed in Table 1.

As stated above, the value obtained for \( K \) cannot be interpreted as the global association constant if the number of molecules per aggregate \( n \) is unknown. An estimate of such number can be provided by the same data, plotting \( \ln(C\Delta) \) as a function of \( \ln(C\Delta_0) \) and fitting by the following curve\textsuperscript{38}:

\[ \ln(C\Delta) = n \ln(C\Delta_0) + \ln K_a + \ln(n) - (n - 1) \ln \Delta_0 \]

where \( \Delta \) is the observed deviation from the shift of the monomer (\( = \delta_{\text{obs}} - \delta_m \); Figure 1c).

Since the fitting is very sensitive to \( \Delta \) and \( \Delta_0 \), we extrapolated the values of \( \delta_m \) and \( \delta_{\text{aggr}} \) (and hence \( \Delta \) and \( \Delta_0 \)) using a hyperbolic function (Figure 1b) mimicking the plot of the chemical shift \( \delta_{\text{obs}} \) as a function of the reciprocal concentration \( (1/C) \). Such plot is also very informative being able to provide the critical concentrations for aggregation which is found at the intersection of the two tangents to the curve as shown in Figure 1b\textsuperscript{39}. We found a critical concentration of \( 8.43 \pm 0.05 \text{ mM} \). As the hyperbolic curve would never cross the vertical axis at 0 concentration, we allowed the curve to shift along the horizontal axis by a quantity \( B \) using the following equation:

\[ \delta_{\text{obs}} = \delta_m + B/(1/C + b) \]

where \( B \) is a parameter shifting the curve along the vertical axis. Regression analysis gave the values of \( \delta_m \) and \( \delta_{\text{aggr}} \) (\( \delta_{\text{obs}} \) for \( 1/C = 0 \)) and consequently \( \Delta \) and \( \Delta_0 \) to be used in Eq. 3.

Figure 1c shows the fitting of Eq. 3 indicating that in the range of concentration used, the predominant species formed with \( K_a = 10 \pm 1.8 \text{ M}^{-1} \) is a dimer \( (n=2) \), in agreement with previous studies\textsuperscript{33,36}. This finding permits

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Solvent</th>
<th>Model</th>
<th>( K ) (M(^{-1}))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>30.5</td>
<td>D(_2)O</td>
<td>Dimerization</td>
<td>5.5–5.6</td>
<td>\textsuperscript{20}</td>
</tr>
<tr>
<td>30</td>
<td>D(_2)O</td>
<td>Isodesmic</td>
<td>8.4±1.3</td>
<td>\textsuperscript{22}</td>
</tr>
<tr>
<td>25</td>
<td>0.1 M phosphate buffer, pD=7.1</td>
<td>Isodesmic</td>
<td>11.8±0.3</td>
<td>\textsuperscript{36}</td>
</tr>
<tr>
<td>25</td>
<td>0.1 M phosphate buffer, pD=7.1</td>
<td>Indefinite cooperative</td>
<td>10.5±0.6</td>
<td>\textsuperscript{36}</td>
</tr>
<tr>
<td>15</td>
<td>H(_2)O/dDMSO 9:1 v/v</td>
<td>Isodesmic</td>
<td>7.4±0.9</td>
<td>\textsuperscript{23}</td>
</tr>
<tr>
<td>27</td>
<td>D(_2)O/dDMSO 9:1 v/v, pH=3.8</td>
<td>Isodesmic</td>
<td>7.9±0.7</td>
<td>\textsuperscript{7}</td>
</tr>
<tr>
<td>27</td>
<td>H(_2)O/dDMSO 9:1 v/v, pH=4±0.5</td>
<td>Isodesmic</td>
<td>6±1</td>
<td>\textsuperscript{8}</td>
</tr>
</tbody>
</table>
to define the global association constant $K_i$ for the isodesmic model as the value obtained for each single step $(7.6 \pm 0.5 \text{ M}^{-1}; \text{Eqs. 2a and 2b}).$

**Chlorogenic Acid Self-Association**

The same set of measurements performed on caffeine were repeated for chlorogenic acid (Figure 2). In this case, we dissolved the molecule in phosphate buffer $80 \text{ mM}$ at pH 7 in order to prevent perturbations of the shifts due to the change in pH as the amount of acid increases.

We found a self-association constant of $K_i = 2.8 \pm 0.6 \text{ M}^{-1}$, the formation of a dimer with a critical concentration of $22.8 \text{ mM}$ and $K_a = 3.4 \pm 0.6 \text{ M}^{-1}$.

These data demonstrate that chlorogenic acid shows a slight decreased tendency to aggregation as compared to caffeine. Several polyphenols, particularly tea components, have been NMR characterized, as far as self-association in aqueous media is concerned. Upon polyphenols dilution, NMR experiments displayed changes in chemical shift of several proton resonances, consistent with stacking interactions of the aromatic moieties. The constant determined for chlorogenate self-association falls within the range reported for methyl gallate and propyl gallate of $2–12 \text{ M}^{-1}$.8,41

**Caffeine–Chlorogenic Acid Complex**

Although the formation of an adduct between caffeine and chlorogenic acids was first reported one century ago,1 NMR in solution as well as solid state studies are far from being abundant. An X-ray structure has been determined in the presence of potassium,14 while Horman and Viani in the '70s12,13 proposed an average complex formation in solution.

In view of the extensive worldwide consumption of coffee and caffeinated beverages and the consequent possible physiological effects, we decided to reinvestigate both the stability and the structural features of such complex.

In order to evaluate the equilibrium constant, we chose to work at a concentration as low as possible in order to have both molecules in monomeric forms.

We titrated a solution of caffeine $0.76 \text{ mM}$ with a concentrated solution of chlorogenic acid following the chemical shift of H$_{11}$ protons of caffeine. Given the equilibrium constants for self-association estimated above, at $0.76 \text{ mM}$, caffeine is almost completely monomeric (99.5%). This is even more valid for chlorogenic acid, whose association constant is lower.

Standard regression analysis was used to fit the experimental data by using the equation (Figure 3a):

$$
\Delta = \Delta_0 \frac{C_{\text{free}}^M}{K_d + C_{\text{free}}^M} \quad (5)
$$

where $K_d$ is the inverse of the association constant for the formation of a M–L complex starting from M and L molecules, $\Delta$ is the actual change of chemical shift of L (= caffeine), $\Delta_0$ is its limiting value when fully complexed, and $C_{\text{free}}^M$ is the experimental chlorogenic acid concentration corrected by using the relation$^{32,43}$:

$$
C_{\text{free}}^M = C_M - C_L \frac{\Delta}{\Delta_0} \quad (6)
$$

where $C_M$ and $C_L$ are the total concentrations of chlorogenic acid and caffeine, respectively.

The value of the association constant ($= 1/K_d$) was calculated to be $30 \pm 4 \text{ M}^{-1}$. Our value substantially agree with what found by Horman and Viani (16.9 M$^{-1}$ at 40°C). Taking into account the different temperature used, despite the much higher concentrations used (which might have result in substantial interferences from self-associations).
In order to structurally characterize the complex, we recorded a series of 2D $^1$H,$^1$H-NOESY spectra to detect intermolecular NOEs. The experiments were performed on a solution 20 mM for both molecules to gain in signal/noise ratio and to reproduce the concentrations commonly found in the beverage. NOEs were found among the caffeoyl portion of chlorogenic acid and caffeine (Figure 3b), indicating a kind of stacking interaction between the aromatic rings. Intermolecular NOEs were converted into distance restraints as described in “Materials and methods” section to generate the structure shown in Figure 3c.

In the complex, the imidazole ring of caffeine is oriented toward the quinic moiety of the chlorogenic acid as in the X-ray structure published by Martin et al. However, NOEs place the caffeine plane rotated by 180° along the longitudinal axis of the molecule. The results substantially differs from the one proposed by Horman and Viani, although both agree in a stacking interaction between the aromatic rings. The structure we report is probably the most abundant in solution and does not necessarily exclude what reported by these authors as demonstrated by the presence of NOEs which are not fully consistent with a unique structure. For example, the weaker NOE between H$_5$′ of chlorogenic acid and H$_{12}$ of caffeine reported in Figure 4b could originate from a smaller fraction of molecules in the orientation proposed by Horman and Viani.

Another important difference is the orientation of the caffeoyl aromatic ring with respect to the double bond in the chlorogenic acid which in our complex is rotated by 180° (in the structure by Horman and Viani H$_8$ points toward H$_6$′ rather than H$_2$′). The intramolecular NOE between H$_2$′ and H$_8$′ unequivocally define their mutual orientation which is
also in agreement with the X-ray structure. However, given the concentrations used and the value of the association constant, only 30% of the molecules are forming the complex and the intramolecular NOE (unlike the intermolecular NOEs) might also arise from the free form of chlorogenic acid.

In order to justify the higher value of the association constant with respect to the self-association constants of both molecules, we looked for specific interactions stabilizing the complex. The hydroxyl in position 4' on chlorogenic acid might interact with the carbonyl in position 2 on caffeine, possibly through a bridging water molecule. Based on the work by Araque et al. on caffeine interacting with oleate, we looked for a possible contact between the carboxylate of the quinic moiety with the imidazole hydrogen of caffeine. Although the two groups can come close up to 0.34 nm, we believe this interaction must be excluded in our complex as we should have found NOEs between H12 and H8 of caffeine and H2 protons of the quinic portion. To this respect, when looking at the structure shown in Figure 3c, one should be aware of the likely high degree of mobility of the quinic moiety.

Caffeine in Espresso Coffee Brew

It is well known that differently from other brewing techniques, conditions normally used to brew espresso coffee enhance several surface tension-related phenomena such as emulsion and foam formation. In particular, the presence of an emulsified lipid fraction makes espresso coffee a very interesting beverage model to assess possible caffeine complexation by compounds different from chlorogenic acids. The molecular structure of caffeine–oleate complex in aqueous systems has been recently reported.

In the present work, espresso coffee brews have been characterized under experimental conditions close to those of Tavares and Ferreira.

In Figure 4, the typical 1H-NMR spectrum of espresso coffee brew is reported. The spectrum is in excellent agreement with that reported by Tavares and Ferreira. In particular, the signals of caffeine, trigonelline, N-methyl piridinium, formic acid, free quinic acid and chlorogenic isomers are present and well resolved. Moreover, in full agreement with Bosco et al., broad signals relative to the methyl and methylene groups of the fatty acids and triglycerides are also evident, confirming the presence of an emulsified lipid fraction. Similar signals were also found in the spectrum reported by Tavares and Ferreira although the authors not evidenced their presence.

By carefully comparing the chemical shifts of the several coffee compounds characterizing the 1H-NMR spectrum, it appears that caffeine and chlorogenic acid chemical shifts are remarkably displaced in comparison with those of their
pure compounds at similar concentrations, as shown in Figure 5.

However, chemical shift differences almost disappear when the mixture is taken as a reference demonstrating that the complex is indeed present in the real beverage.

Conclusions

The present NMR re-investigation confirms some previous findings on caffeine self-association and caffeine–chlorogenic acid complex. Equilibrium constants are in full agreement with previous studies and a stacking interaction in the formation of the complex is further verified. Additionally, the self-association of chlorogenic acid is reported for the first time and the structural features of the complex with caffeine are revisited using intermolecular NOE effect. The comparison between NMR spectra of the mixture of the two pure molecules with that of espresso coffee brew shows that unlike the pure components, the mixture is able to fully reproduce the real system.

Based on the equilibrium constant and the concentration used in the mixture (20 mM for both molecules), we can estimate a molar fraction of bound caffeine of about 30%.

Fig. 5 ¹H-NMR spectra of caffeine (20 mM), chlorogenic acid (20 mM, pH 7), 1:1 caffeine–chlorogenic acid mixture (20 mM, pH 7), and espresso coffee brew. In the insert, the region of N-methyl groups of caffeine is magnified.
Accordingly, a similar amount is needed to reproduce the real spectrum (the shift with respect to the pure caffeine for H1 is about 0.12 ppm while the limiting shift in case of 100% complexation should be 0.3 ppm as derived from fitting of Eq. 5). However, while caffeine content in espresso coffee fall within these range of concentrations needed (15–20 mM), chlorogenic acid is present in a lower amount (10 mM) accounting for 20% of binding. We can therefore conclude that caffeine is probably bound to other species in solution giving rise to similar effects on its chemical shift. It is noteworthy that the integral of H8 of caffeine is very close to that of the signals between 7.3 and 7.6 ppm in which H7 of chlorogenic acid (and of all similar molecules) fall. It can be therefore hypothesized that feruloylquinic acid as well as dicaffeoyl- and diferuloylquinic acids and related lactones be therefore hypothesized that feruloylquinic acid as well as dicaffeoyl- and diferuloylquinic acids and related lactones formed on roasting may contribute to complex formation with caffeine. Additionally, it has been reported that a small fraction of chlorogenic acid can be covalently bound to green coffee proteins; these species, still present in roasted coffee as melanoids, may provide an additional source of chlorogenic acid (not visible in the NMR spectrum because of the large molecular weight). In this scenario, the physiological role played by caffeine complexation with polyphenol yields in coffee brews might be the subject of additional studies.

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