Potential Use of a Terbium-Transferrin Complex as a Label in an Immunoassay for Gentamicin

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A study has been made of the potential use of a terbium - transferrin complex as a non-isotopic label in the immunoassay determination of the antibiotic gentamicin. The fluorescence properties of the complex have been characterised. The labelled gentamicin was formed using a controlled carbodiimide reaction, conditions being chosen to produce a gentamicin-bound complex containing the correct amount of gentamicin for use in a competitive binding assay. Recognition of the gentamicin-bound complex by antisera to gentamicin was verified using a standard radioimmunoassay for gentamicin.

Keywords: Fluorescence immunoassay; gentamicin assay; terbium - transferrin label

Reagents
Terbium chloride and glycine were obtained from Aldrich Chemical Co. and iron-free transferrin, gentamicin sulphate and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride from Sigma London Chemical Co.
All radioimmunoassays were carried out using a radioimmunoassay kit supplied by RIA (UK) Ltd.
Antibodies used in the antibody binding experiments were supplied by Sigma Chemical Co. Ltd.

Fluorescence of Terbium and the Terbium - Transferrin Complex
Fluorescence spectra of terbium chloride in aqueous solution and a terbium - transferrin complex in Tris - hydrochloric acid - hydrogen carbonate buffer were recorded.
The limits of detection of the complex and its fluorescence lifetime (the time for the fluorescence of a sample to decay to the background level) were also determined.
The complex for the above experiments was prepared by the addition of aqueous terbium chloride to iron-free transferrin in Tris - hydrochloric acid - hydrogen carbonate buffer in the molar ratio 2:1.

Linkage of Gentamicin to Transferrin
The linkage of gentamicin to transferrin was achieved using a carbodiimide reagent. In this procedure a free acid group on the transferrin molecule is linked to a free amino group on the protein fraction on a Sephadex G-25 column by elution with Tris - hydrochloric acid - hydrogen carbonate buffer (pH 8.5). Verification of gentamicin binding was carried out using 125I-labelled gentamicin.
The carbodiimide procedure was repeated using various glycine to gentamicin ratios for the gentamicin in the original experiment, the reaction time being 5 min in each instance. The reaction products were subsequently analysed for their gentamicin content by radioimmunoassay and their fluorescence was measured after terbium binding.
Results and Discussion

Fluorescence of Terbium and the Terbium - Transferrin Complex

Fig. 1 shows an enhancement of the fluorescence intensity of terbium of the order of 10^5 when the complex is excited at 295 nm. This fluorescence enhancement is due to an intramolecular energy transfer process within the complex. This is demonstrated by the fact that the complex excitation is in the region of the ligand absorbing region whereas the emission is typical of the "line" emission of the terbium ion.

The limit of detection of the complex was shown to be 10^{-7} M with respect to transferrin, a result which suggests that the complex has a suitable fluorescence intensity for use as a label in the analysis of drugs by immunoassay.

The complex had a lifetime of 1.25 ms. Subsequent delayed fluorescence experiments (Fig. 2) showed that interference due to scattered light from blood serum could be reduced to zero with a delay gate of 0.05 ms. The subsequent decrease in fluorescence intensity of the complex was less than 5%. The following instrumental conditions were thus chosen: excitation slit, 5.0 nm; emission slit, 5.0 nm; excitation wavelength, 295 nm; delay time, 0.05 ms; gate time, 5.0 ms; and fixed-scale mode.

Analysis of the Gentamicin Content of Complexes with Substituted Glycine

Table 1 shows the amount of gentamicin bound per millilitre of solution for various reaction mixtures as analysed by standard radioimmunoassay for gentamicin.

It can be seen that gentamicin - glycine bound complexes with glycine to gentamicin ratios of less than 15:5 have too high a gentamicin content for use in a competitive binding assay for the therapeutic blood concentration range of gentamicin (4-12 µg ml^{-1}).

Fluorescence measurements on the complexes modified by glycine and gentamicin showed a decrease in fluorescence intensity of less than 5% compared with the original terbium - transferrin complex.

Stability of the Gentamicin - Glycine Bound Complex

It can be seen from Table 2 that a complex containing a glycine to gentamicin ratio of 9:1 is stable in buffer and buffer containing 1:100 human serum over a period of at least 7 d.

Absorbance measurements at 295 nm showed that the decrease in fluorescence intensity in 1:100 serum was due to the increased absorbance of the solution at that wavelength.

Analysis of the Gentamicin Content of Complexes with Substituted Glycine

Table 1. Results of analysis of complex for gentamicin by radioimmunoassay

<table>
<thead>
<tr>
<th>Analyte</th>
<th>C. p.s.</th>
<th>Gentamicin determined/µg ml^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µg ml^{-1} standard</td>
<td>42039</td>
<td>&gt;16</td>
</tr>
<tr>
<td>1 µg ml^{-1} standard</td>
<td>36557</td>
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<td>2 µg ml^{-1} standard</td>
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<td>4 µg ml^{-1} standard</td>
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<tr>
<td>8 µg ml^{-1} standard</td>
<td>9860</td>
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<tr>
<td>16 µg ml^{-1} standard</td>
<td>6481</td>
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<tr>
<td>Glycine : gentamicin ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0:20</td>
<td>1023</td>
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<tr>
<td>2:18</td>
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<td>18:2</td>
<td>11493</td>
<td>4-8</td>
</tr>
</tbody>
</table>

Linkage of Gentamicin to Transferrin

The experiments carried out using 125I-labelled gentamicin showed that with reaction times from 5 min to 1 h substantial gentamicin binding was occurring.

Analysis of the Gentamicin Content of Complexes with Substituted Glycine

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Titration of the Glycine - Gentamicin Complex against Anti-gentamicin

Titration of the complex against anti-gentamicin (Fig. 3) shows good antibody recognition of the complex, the curve shape being typical of such a dilution analysis.
Conclusion

The results suggest that a glycine - gentamicin bound terbium - transferrin complex has potential as a non-isotopic immunoassay label. However, it is felt that careful consideration should be given to the final format of the immunoassay, owing to possible effects of the macromolecular label. At present the most suitable separation methods are being established.

Although the terbium - transferrin complex shows potential as an alternative label in immunoassay, it has the disadvantage of sensitivity suitable only for the detection of drugs and not hormones. One means of increasing the sensitivity is to increase the fluorescence intensity. Further study of the intramolecular energy transfer process\(^7\)\(^8\) such as are involved in the terbium - transferrin complex might prove useful in this respect. The bifunctional reagents that would be suitable are of the type described by Meares and co-workers,\(^9\)\(^10\) which have “EDTA” groups and groups suitable for binding to amino acids at opposite extremities of the molecule. Another alternative is the use of bifunctional reagents where the chelating agent is “iminodiacetic acid.”\(^11\)\(^12\)

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References