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# A Quantitative Spectrometric Assay for the Determination of Solution Concentration of *para*-sulphonato-calix[*n*]arenes and Their Derivatives

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**Abstract**

A spectrometric assay for the determination of concentration of *para*-sulphonato-calix[*n*]arenes and their derivatives has been developed using dimethylmethylene blue (DMMB) as a probe. Interaction with *para*-sulphonato-calix[*n*]arenes leads to a metachromatic shift in the spectrum of DMMB with appearance of a peak at 536 nm and diminution of the spectral intensity of the peaks at 594 and 649 nm. The method shows good linearity in the concentration range 0–6 $\mu$g/ml for *para*-sulphonato-calix[*n*]arenes.

## Introduction

During the last few years, there has seen increasing interest in the biological properties of the calix[*n*]arenes [1], and in particular the *para*-sulphonato-calix[*n*]arenes. These highly water-soluble derivatives have demonstrated a wide spectrum of biological activities; ranging from ion-channel blocking [2], anti-thrombotic properties [3], anti-viral activity [4] to enzyme inhibition [5]. Numerous fundamental studies have been dedicated to understanding their activity, using amino acids as probes to determine how the calix[*n*]arenes bind to proteins [6–8].

A strong analogy with the activity of the glycosaminoglycans [9], including heparin, chondroitin sulphate and dermatan sulphate is emerging. Given the ubiquitous nature of the biological activity of the glycosaminoglycans, we expect an even wider range of biological activity to emerge for the *para*-sulphonato-calix[*n*]arenes. Such activity necessitates a reliable method for assaying the concentration of the *para*-sulphonato-calix[*n*]arenes. This is in contrast to the normal use of supramolecular systems as sensor molecules.

In this paper, we present a spectrometric method for the determination of the solution concentrations of the *para*-sulphonato-calix[*n*]arenes and a number of their derivatives. The method is based on the use of 1,9-dimethylmethylene blue (DMMB), a spectrometric reagent previously used in the assays for the glycosaminoglycans [10, 11].

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## Experimental

### *Polyanionic molecules*

Different polyanionic molecules, see Figure 1 for the chemical structures, were studied according to a method developed from that established by Farndale to quantify sulphated glycosaminoglycans [10, 12]. Chondroitin sulphate, **1** (sodium salt from shark cartilage, type C, Sigma) was used as a standard. Other polyanionic molecules including: Heparin, **2a** (heparin potassium salt from porcine intestinal mucosa Fluka); Heparin, **2b** (heparin calcium salt from hog intestinal Fluka); Pentosan polysulphate (Sigma) **3**; poly(sodium 4-styrene sulphonate) (Aldrich), **4**, were studied. HBS (4-hydroxybenzene sulphonic acid) (Aldrich), **5**, was used as a monomeric standard equivalent to a single residue of the *para*-sulphonato-calix[*n*]arenes, **6**, **7**, **8**.

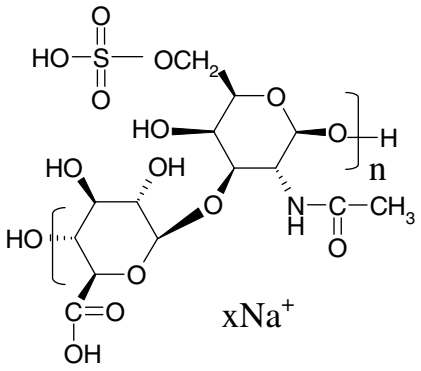
### *Synthesis of the* para-*sulphonato-calix[*n*]arene derivatives*

All *para*-sulphonato-calix[*n*]arenes, (see Figure 1), (**6a**, **7a**, **8a**) and their derivatives either the mono-O-methoxycarboxylic acid forms (**6b**, **7b**, **8b**), or the mono-O-ethoxyamine forms (**6c**, **7c**, **8c**) were synthesised according to the literature methods, and their physical properties are in full agreement with previously published data [6].

### *Sample preparation*

Polyanion solutions were prepared by dissolving molecules at a concentration of 0.5 mg/ml in distilled water.

1:

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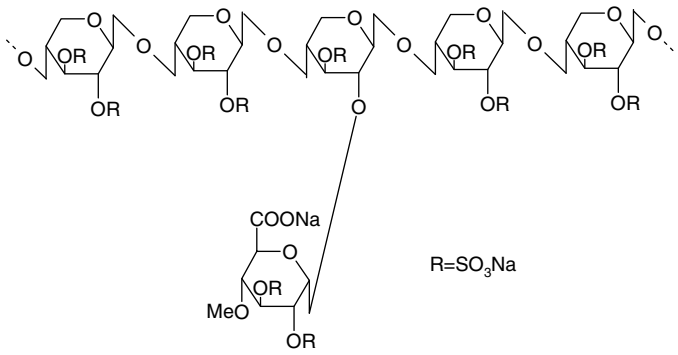


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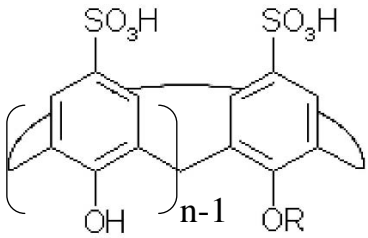


*Figure 1.* Molecular structure of 1: chondroitin sulphate, 2: pentosan polysulphate and *para*-sulphonato-calix[*n*]arenes 3: $n = 4$, 4: $n = 6$, 5: $n = 8$; and a: R=H, b: R=$CH_2COOH$ mono-O-methoxycarboxylic acid form, c: R=$CH_2NH_2$, mono-O-ethoxyamine form.

Solutions of the various *para*-sulphonato-calix[*n*]arene derivatives were prepared by dissolving the molecules at a concentration of 0.5 mg/ml in distilled water.

As per the procedure of Farndale, the DMMB reagent was prepared by dissolving in Milli-Q water (>18 MΩ cm), 3.04 g/l glycine (Canalco), 2.37 g/l NaCl (Sigma) and 16 mg/l 1,9-dimethylmethylene blue (DMMB) (Aldrich), solution pH adjusted by HCl, to 3.0 [10]. The reagent is stable if protected from light at room temperature, for 1 month.

*Colorimetric reaction*

Samples were prepared in Tris–HCl (Sigma) buffer 10 mM pH 8.0 at a final volume of 80 $\mu$l. Dilutions, corresponding to 0–25 $\mu$g of molecules, were directly prepared in spectrophotometric micro-cells to avoid mixing. One millilitre of colour reagent was added to each assay and the absorbance was immediately read at 525 nm with Beckman UV-DU640 spectrophotometer. The blank was the colour reagent. The experiment was carried out with five independent assays for each sample.

The different *para*-sulphonato-calix[*n*]arene derivative samples were diluted in the same way as described above, at 1.8 $\mu$g/ml in order to scan wavelengths from 450 to 700 nm and compared to the colour reagent. The blank was distilled water.

## Results and discussion

The structures of the calix[*n*]arenes and the various glycosaminoglycans and polyanionic polymers used in this work are given in Figure 1. The structural analogy between the *para* sulphonato-calix[*n*]arenes and the glycosaminoglycans is based on the presence of sulphonate or sulphate functions, the presence of a 'hydrophobic' six membered ring, phenolic for calix[*n*]arenes and hexose for the glycosaminoglycans, hydroxyl groups providing hydrogen banding sites. In general, the dimeric repeat

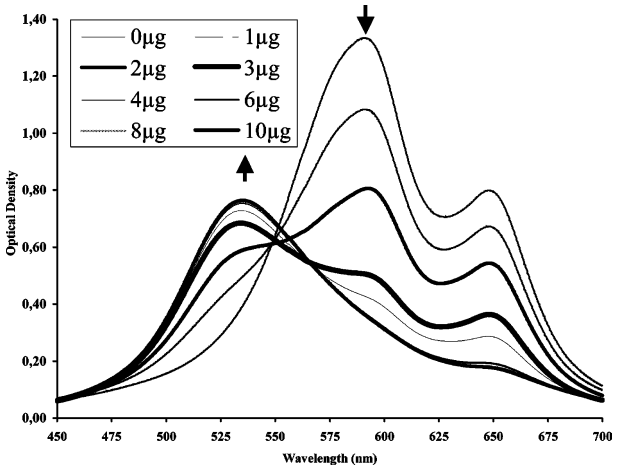


*Figure 2.* Spectrometric titration curves for DMMB in the presence of increasing quantities of **7a**.

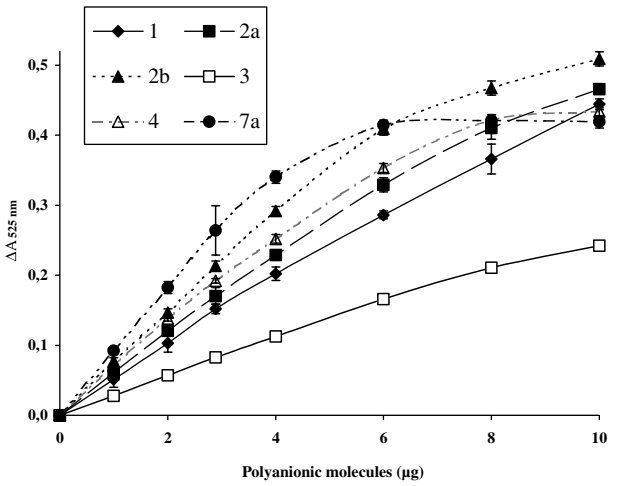


*Figure 3.* Optical response curves for polyanionic molecules with DMMB reagent. Chondroitin sulphate was used as standard. Polyanionic molecules standard solutions were prepared at 0.5 mg/ml in water. Increasing quantities of polyanions (from 0 to 10 $\mu$g) were diluted in a final volume of 80 $\mu$l with Tris – HCl buffer 10 mM pH 8.0. One millilitre of DMMB reagent was added and optical density was immediately read at 525 nm. $n = 5$.

unit of the glycosaminoglycans is comparable in size to two phenolic units in the calix[*n*]arenes.

In Figure 2, are presented titration curves for DMMB in the presence of increasing concentrations of *para*-sulphonato-calix[6]arene, **7a**; as expected a strong metachromatic effect is observed. [13]. The peaks at 594 nm and 649 nm, resulting from the presence of the dimeric form of DMMB, in solution decrease in intensity, while an absorption peak at 536 nm, arising from the complexed monomeric form of DMMB appears and increases in intensity with increasing concentrations of **7a**. No significant variations in the wavelengths of the absorption maxima are observed for all systems studied here.

The lack of a single isobestic point implies that the complexation process involves more than one species.

Polyanionic molecules were studied by a colorimetric method using the DMMB reagent, allowing obtention of calibration curves after reading solution absorbance at 525 nm. Concentration-dependent optical response plots for various polyanionic molecules are shown in Figure 3. In view of the highly polydisperse nature of the natural polysaccaharides, we consider the use of molarities to be uninformative and use weight of added anion. A significant linear response ($R^2 = 0.9953$) is obtained from 0 to 10 $\mu$g chondroitin sulphate. The useful range of the assay cannot be extended up to 10 $\mu$g because of some deviation from linearity. The same is true for all studied polyanionic molecules but the linearity limits differ according to the molecules: 6 $\mu$g for heparins (**2a** and **2b**) and pentosan polysulphate (**4**), 10 $\mu$g for PSS (**3**). Results are summarised in Table 1.

This spectrophotometric method shows a significant, linear and reproducible response for all *para*-sulphonato-calix[*n*]arenes (**6a**, **7a**, **8a**) and their derivatives; the mono-O-methoxycarboxylic acids (**6b**, **7b**, **8b**) and the mono-O-ethoxyamines (**6c**, **7c**, **8c**). In Figure 4 are given the optical response curve for **6a**, **7a**, **8a** and **5**. Importantly, it can clearly be seen that the 'monomer' **5** (HBS) gives no modification in the spectrum of DMMB. A high reproducibility of this method is observed, as the standard deviations are minor, less than 5%.

The linearity limits for the *para*-sulphonato-calix[*n*]arenes and their derivatives are in the concentration range of 3–6 $\mu$g/ml. With regard to the colorimetric response, i.e. the change in DO at 525 nm, the results are summarised in Table 1.

The colorimetric response is dependent on the ring size with *para*-sulphonato-calix[6]arene derivatives, **7a**, **7b**, **7c**, greater than the corresponding *para*-sulphonato-calix[4]arene derivatives, **6a**, **6b**, **6c** and than *para*-sulphonato-calix[8]arene derivatives, **8a**, **8b**, **8c**, in all cases. For a given macrocyclic ring size the colorimetric response depends on the nature of the substituent function at the hydroxyl face. For a calix[*n*]arene quantity of 2 $\mu$g, the colorimetric response is higher for **6a** ($\Delta A_{525\ nm} = 0.1540$) than for **6c**($\Delta A_{525\ nm} = 0.1266$),

*Table 1.* Optical responses at 525 nm for 2 $\mu$g of various polyanionic molecules and maximal linearity limits

| Polyanionic molecules | Maximal linearity limits ($\mu$g) | $\Delta A_{525\ nm}$ for 2 $\mu$g |
|---|---|---|
| **1** | 10 | 0.1032 |
| **2a** | 6 | 0.1204 |
| **2b** | 6 | 0.1465 |
| **3** | 10 | 0.0570 |
| **4** | 6 | 0.1380 |
| **6a** | 6 | 0.1540 |
| **7a** | 4 | 0.1826 |
| **8a** | 6 | 0.0461 |
| **6b** | 6 | 0.1097 |
| **7b** | 6 | 0.1544 |
| **8b** | 3 | 0.1470 |
| **6c** | 6 | 0.1266 |
| **7c** | 3 | 0.2545 |
| **8c** | 3 | 0.2024 |

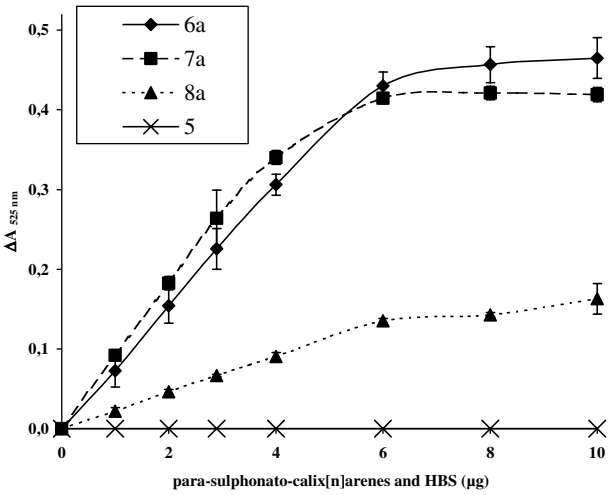


*Figure 4*. Calibration curves for polyanionic molecules: **6a**, **7a**, **8a** and **5**. Polyanionic molecules standard solutions were prepared 0.5 mg/ml in water. Increasing quantities of polyanions( from 0 to 10 $\mu$g) were diluted in a final volume of 80 $\mu$l with Tris–HCl buffer 10 mM pH 8.0. One millilitre of DMMB reagent was added and optical density was immediately read at 525 nm. ($n = 5$).

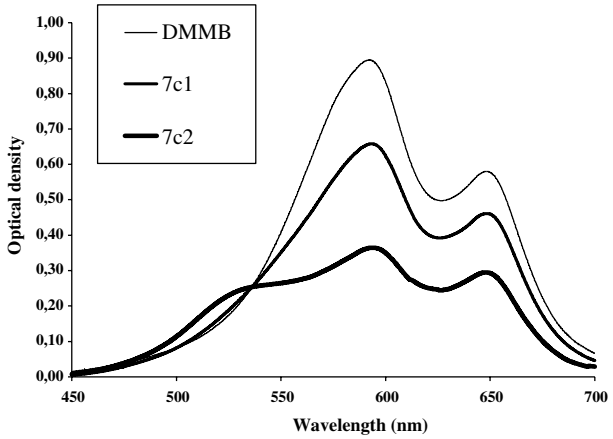


*Figure 5*. Compared spectra of two samples of para-sulphonato-calix[6]arene (**7c1** and **7c2**), with DMMB reagent spectrum. **7c1** and **7c2** can be distinguished by their degree of purity. Standard solutions were prepared in 0.5 mg/ml in water and diluted to 1.8 $\mu$g/ml in a final volume of 80 $\mu$l with Tris – HCl buffer 10 mM pH 8.0. One millilitre of DMMB reagent was added and wavelenghts were immediately scanned from 450 to 700 nm.

and the response obtained for **6b** is the lowest of these three chemical forms ($\Delta A_{525\ nm} = 0.1097$). In contrast, in the case of the calix[6]arene derivatives, for a calix[*n*]arene quantity of 2 $\mu$g, the colorimetric response is higher for **7c** ($\Delta A_{525\ nm} = 0.2545$) than for the **7a** ($\Delta A_{525\ nm} = 0.1826$). Finally in the calix[8]arene series, **8c** carrying an amine function shows a higher response than **8b** and much higher than the parent compound **8a**. Hence both ring size and substituent nature influence the linearity range and the colorimetric response. This undoubtedly arises from the strength of complexation of DMMB by the calixarenes. Due to the complexity of the association process as noted above, no single isobestic point is observed, the association constants have not been determined.

A second factor for determining the linearity range and colorimetric response lies in the modification of the geometry of the higher calixarenes by monosubstitution [14], which will modulate the presentation of both the hydrophobic binding pocket and the sulphonate groups towards DMMB.

In order to study whether the method is applicable to analysis of the purity of the *para*-sulphonato-calix[*n*]arenes, spectra were obtained from 450–700 nm for the DMMB colour reagent alone and for two samples of **7c**, differing only in their degree of purity. The visible absorption spectrum of DMMB reagent (see Figure 5) shows two maxima at 594 nm and at 649 nm. Addition of a quantity of **7c** results in a decrease in the intensities of both peaks, with no shift in their wavelengths of maximum absorption (Figure 5). In addition, a third band at 536 nm appears in presence of **7c**. This third band at 536 nm increases in intensity with increasing quantities of both samples of **7c**. The difference in the decrease in intensities of the peaks at 594 nm and 648 nm and the increase in the intensity of the 536 nm peaks, for the same weight of **7c**, must arise from differences in the real concentration of the calixarene. Both samples of **7c** show only [M + $Na^+$] peaks in the ES/MS spectrum, however sample 2, shown here to have a higher degree of purity was a fraction collected from a narrower elution time band in the RP-HPLC purification of the samples.

Selectivity of the assay for *para*-sulphonato-calix[*n*]arenes is therefore greatly improved, and it is for example, possible to quantify *para*-sulphonato-calix[*n*]arenes in production. Analysis of the spectra shows that 594 nm would appear to be the most adequate wavelength to discriminate *para*-sulphonato-calix[*n*]arenes in a complex mixture (without sulphated glycosaminoglycans).

## Conclusion

In conclusion, we have developed a useful spectrometric assay for the determination of concentrations of *para*-sulphonato-calix[*n*]arenes and their derivatives, in concentration range 0–6 $\mu$g/ml. The results show response curves dependent on both the macrocyclic ring size and the nature of substitution at lower rim.

## Acknowledgment

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## References

1. E. Da Silva, A.N. Lazar, and A.W. Coleman: *J. Drug Deliv. Sci. Tech.* **14**, 3 (2004).
2. J.L. Atwood, R.J. Bridges, R.K. Juneja, K. Ravindra, and A.K. Singh: *US Patent 748764* (1996).
3. K.M. Hwang, Y.M. Qi, S.Y. Liu, T.C. Lee, W. Choy, and J. Chen: *US Patent 5409959* (1995).

4. K.M. Hwang, Y.M. Qi, S.Y. Liu, W. Choy, and J. Chen : *US Patent 5441983* (1995).
5. D.J.S. Humes, A.W. Coleman, and E. Aubert-Fouchet: *French Patent FR 2782007* (1998).
6. E. Da Silva and A.W. Coleman: *Tetrahedron* **59**, 7357 (2003).
7. G. Arena, A. Contino, F.G. Gulino, A. Magrì, F. Sansone, D. Sciotto, and R. Ungaro: *Tetrahedron Lett*. 40 (8), 1597 (1999).
8. L. Memmi, A.N. Lazar, A. Bioude, V. Ball, and A.W. Coleman: *J. Chem. Soc.Chem. Commun*. **2474**, 125 (2001).
9. M. Yalpani: *Polysaccharides*, Elsevier, New York (1988).
10. R.W. Farndale, D.J. Buttle and A.J. Barrett: *B.B.A* **883**, 173 (1986).
11. D.M. Templeton: *Connect Tissue Res*. **17**, 23 (1988).
12. B.O. Enobakhare, D.L. Bader, and D.A. Lee: *Anal. Biochem*. **243**, 189 (1996).
13. (a) E. Robinowitch and L.F. Epstein: *J. Am. Chem. Soc*. **63**, 64 (1941); (b) A.K Panda and A.K Chakraborty: *J. Photochem. Photobiol. A: Chem*. **111**, 157 (1997).
14. E. Da Silva, L. Memmi, A.W. Coleman, B. Rather, and M.J. Zaworotko, *J. Supramol. Chem*. **1**, 135 (2001).