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USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO STUDY THE CAERULOPLASMIN-CATALYSED OXIDATION OF BIOGENIC AMINES

II. MULTIPLE SUBSTRATE SYSTEMS

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SUMMARY

Factors affecting the chromatographic behaviour of the biogenic amines, adrenaline, noradrenaline, dopamine and 5-hydroxytryptamine, and the products of their caeruloplasmin-catalysed oxidation are discussed. These findings are then used to develop an isocratic separation of these eight compounds within a limited time period. This permits the study of the oxidation of biogenic amine mixtures by caeruloplasmin, which cannot be done by conventional enzyme assay methods such as spectrophotometry or polarography. Finally, the results obtained from the oxidation of a mixture of all four amines by caeruloplasmin are presented and discussed.

INTRODUCTION

It has been suggested that caeruloplasmin, a copper-containing protein with oxidase activity, may play a rôle in the regulation of biogenic amine levels within the brain[1]. In a previous paper[2], it has been demonstrated that high-performance liquid chromatography (HPLC) is a valuable technique for following the oxidation of the individual biogenic amines (adrenaline, noradrenaline, dopamine and 5-hydroxytryptamine) by caeruloplasmin. Observations made during this work supported the views of several authors who have suggested that there may be more than one site on the enzyme, whether binding or catalytic, involved in amine oxidation[3,4]. If caeruloplasmin does play a rôle in the regulation of biogenic amine metabolism, there may be interactions between these sites. Therefore, it would be of value to have an assay procedure capable of monitoring the simultaneous oxidation of two or more substrates. This is not possible using existing polarographic or spectrophotometric techniques as these cannot distinguish between the oxidation products of separate amines, but HPLC has the capability to make these distinctions. In order to produce a method that would permit the study of several different substrate mixtures in a single analytical run, it was considered desirable to completely separate the four amines and

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their respective oxidation products within such a time that the most strongly retained peak had a capacity factor (k') of no greater than 12. Adrenaline and adrenochrome have recently been separated on an alkyl phenyl column using a mobile phase of aqueous methanol[5], although this system is unlikely to be sufficiently flexible to achieve the separation criteria described above. The chromatographic conditions used in the previous paper[2] were unsuitable since they were designed to separate each amine from its oxidation product in as short an analysis time as possible. However, similar systems employing an ODS-column, methanol–phosphate buffer and an ion-pairing agent have been used successfully for the separation of the biogenic amines[6] and it was felt that such conditions could be modified to incorporate the products of the caeruloplasmin-catalysed oxidation of these amines.

This paper describes the variables affecting the retention of these compounds on a reversed-phase column, and how these were used to select a mobile phase capable of producing the desired separation. Finally, the results of incubating caeruloplasmin with a mixture of all four amines are briefly discussed.

EXPERIMENTAL

Materials and equipment

Adrenaline, noradrenaline, dopamine, 5-hydroxytryptamine (5HT) and human caeruloplasmin were obtained from Sigma (Poole, Great Britain). Stock solutions of the amines ($5 \cdot 10^{-2}$ *M*) were prepared in 10^{-2} *M* hydrochloric acid and were stable for 4 weeks at 4°C. The aminochromes were prepared each day by incubating buffered solutions of the native amines with caeruloplasmin under the conditions described previously[2]. These solutions were stable for 6 h, on ice, once the oxidation had been terminated with sodium azide. Dilutions of human caeruloplasmin were prepared in 0.25 *M* sodium chloride solution immediately before use. Methanol, acetonitrile, tetrahydrofuran and sodium heptanesulphonate were from Fisons (Loughborough, Great Britain). All other chemicals were purchased from BDH (Poole, Great Britain) and were of analytical grade.

The chromatographic equipment was as described previously[2], with detection at 300 nm used throughout.

Procedure

The constituents of the mobile phase chosen to effect the desired separation of amines and aminochromes on a C_{18} reversed-phase column were (a) a buffer, (b) an organic modifier and (c) an ion-pairing agent.

A series of experiments were designed to demonstrate first, the most suitable candidate for each constituent, and secondly, the concentration required to achieve the desired separation.

A mixture of the eight compounds was prepared daily by individually oxidising $1.25 \cdot 10^{-3}$ *M* solutions of each amine with diluted caeruloplasmin (250 mg/l) and combining these solutions after the oxidation had been stopped with azide.

RESULTS AND DISCUSSION

Buffer selection

The retention characteristics of the amines and their oxidised products were

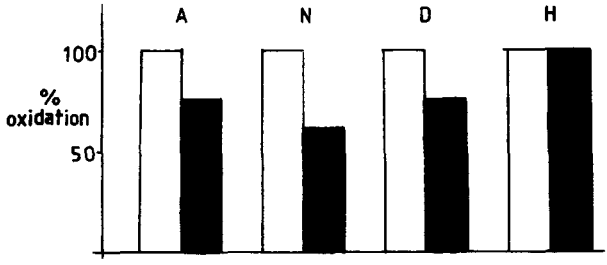

Fig. 1. Comparison of amine oxidation rates in acetate (unshaded) and phosphate (shaded) buffers. A = Adrenaline; N = noradrenaline; D = dopamine and H = 5-hydroxytryptamine. Reaction conditions are as described in a previous paper[2].

unaffected by changes in pH between 4.8 and 6.0. Therefore a buffer pH of 5.5 was chosen as this had been shown to be most suitable for aminochrome stability.

Acetate buffer permitted more efficient oxidation of catecholamines by caeruloplasmin than did phosphate buffer (Fig. 1) and was therefore chosen as the buffer to be included in the incubation mixture. However, the presence of acetate ions in the mobile phase was associated with poor peak shape, possibly due to formation of a complex between the amines and the ions. The peak shape problem did not occur when $5 \cdot 10^{-2}$ *M* phosphate buffer was used in the mobile phase.

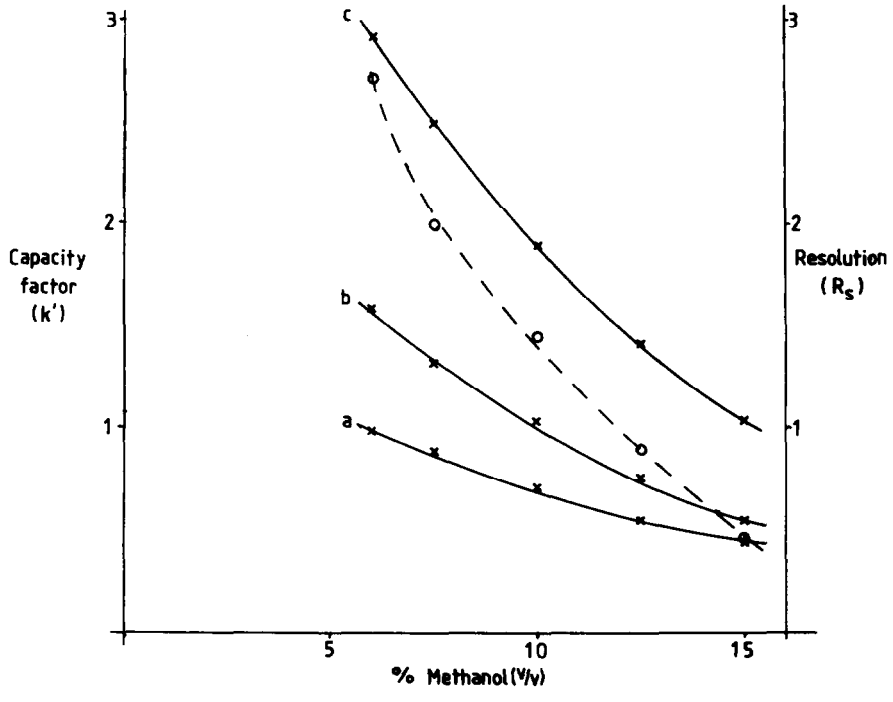

Fig. 2. The effect of methanol concentration on the retention of noradrenochrome (a), adrenochrome (b) and dopaminochrome (c) on a 100 × 4.6 mm I.D. column of ODS-Hypersil; the mobile phase also contained $5 \cdot 10^{-2}$ *M* phosphate buffer (pH 5.5); flow-rate 1.0 ml/min. The broken line represents the calculated resolution (R_s) between the noradrenochrome and adrenochrome peaks.

Organic modifier selection

In reversed-phase chromatography the capability of increasing solute retention by decreasing organic modifier concentration can be coupled with more subtle changes in selectivity achieved by using different modifiers, either alone or in mixtures. In this study, three organic modifiers were compared for their suitability. The presence of tetrahydrofuran in the mobile phase drastically reduced the retention of the aminochromes, and even at very low concentrations of tetrahydrofuran it was impossible to separate adrenochrome from noradrenochrome. The use of this modifier was thus discontinued, leaving a choice of methanol or acetonitrile, with methanol being preferred because of its lower toxicity.

Ion-pairing agent selection

Compounds containing an ionisable amino group can be chromatographed more successfully by ion-pairing with an oppositely charged molecule, thus improv-

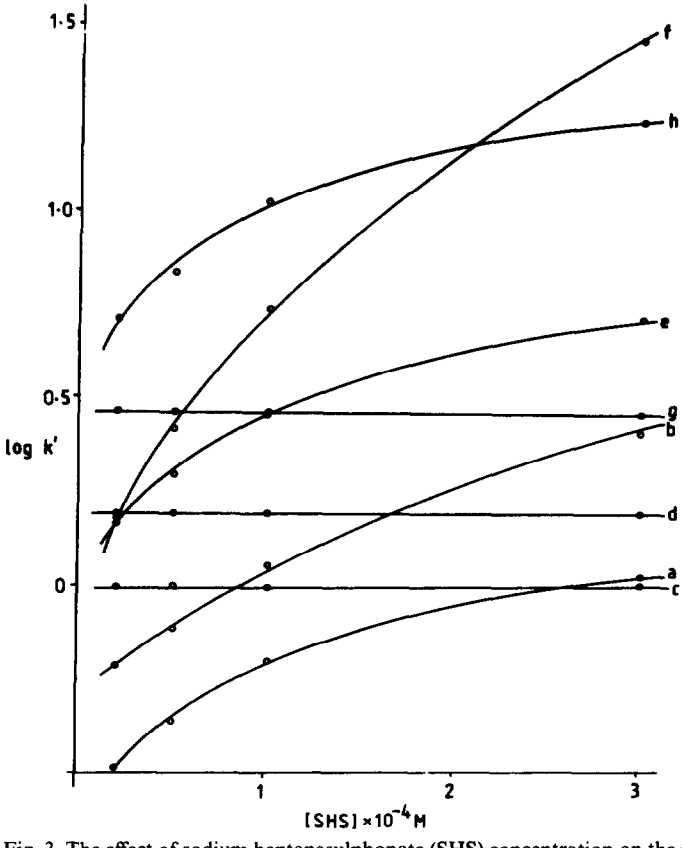

Fig. 3. The effect of sodium heptanesulphonate (SHS) concentration on the retention of noradrenaline (a), adrenaline (b), noradrenochrome (c), adrenochrome (d), dopamine (e), oxidised 5-hydroxytryptamine (f), dopaminochrome (g) and 5-hydroxytryptamine (h) on a 100 × 4.6 mm I.D. column of ODS-Hypersil; the mobile phase also contained 6% (v/v) methanol and $5 \cdot 10^{-2}$ *M* phosphate buffer (pH 5.5); flow-rate 1.0 ml/min.

ing peak shape and increasing retention. Of the eight compounds in this study, the four amines and the oxidation product of 5HT fall into this category. The remaining three aminochromes do not possess a free amino group and their retention is therefore unaffected by changes in mobile phase ion-pairing agent concentration. Work on the estimation of plasma catecholamines in this laboratory has demonstrated the importance of the chain-length of the ion-pairing agent for the separation of these compounds[7], concluding that sodium heptanesulphonate is the most suitable substance for this purpose.

Optimisation of separation

Having established that the final mobile phase should contain phosphate buffer (pH 5.5), methanol and sodium heptanesulphonate, the next stage was to optimise the concentration of each.

In the study of the oxidation of the individual amines[2], $5 \cdot 10^{-2}$ *M* potassium phosphate buffer was used and this has been retained for the current work.

As stated previously, the retentions of the three oxidation products of the catecholamines are unaffected by the concentration of ion-pairing agent. It was therefor decided to determine the effect of methanol concentration on these amino-

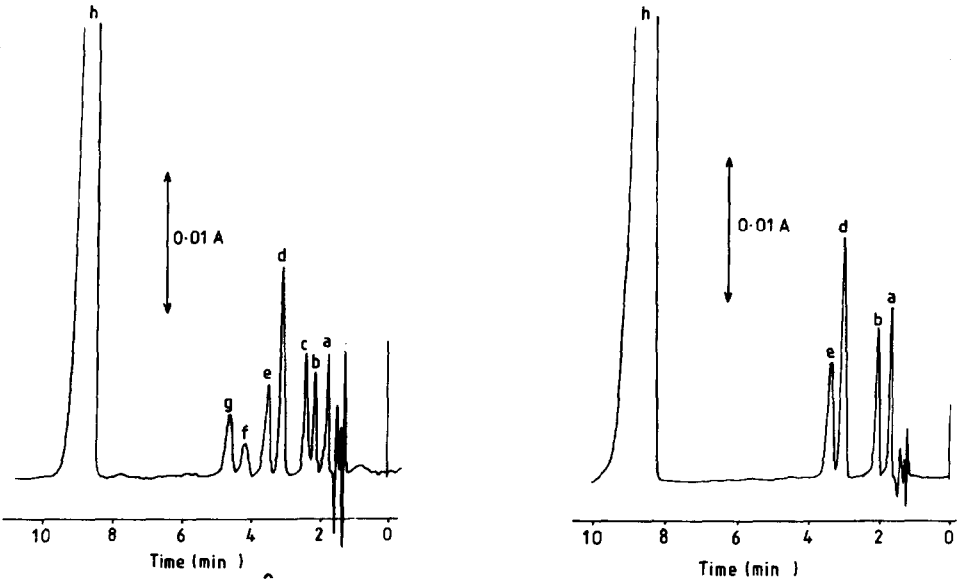

Fig. 4. Chromatogram of the four amines and their oxidised products obtained after combination of incubation mixtures following individual oxidation of each amine. Column: 100 × 4.6 mm I.D. containing ODS-Hypersil; the mobile phase was $5 \cdot 10^{-2}$ *M* phosphate buffer (pH 5.5) containing 6% (v/v) methanol and $4.5 \cdot 10^{-5}$ *M* sodium heptanesulphonate; flow-rate 1.0 ml/min. Detection 300 nm; sensitivity 0.05 a.u. Peak identification as in Fig. 3.

Fig. 5. Chromatogram obtained following the incubation of caeruloplasmin with a mixture of adrenaline, noradrenaline, dopamine and 5-hydroxytryptamine. Conditions as in Fig. 4. Peak identification as in Fig. 3.

chromes and select a modifier level at which the three peaks were well resolved, *i.e.*, resolution (R_s) was greater than 2.0, where:

$$R_s = \frac{\text{difference in retention times}}{\text{mean peak width at base}}$$

The results of this are shown in Fig. 2 from which a methanol concentration of 6% (v/v) was chosen.

Having established the retentions of the three oxidation products, the positions of the remaining five peaks relative to these could be adjusted by altering the concentration of sodium heptanesulphonate.

A series of mobile phases were prepared, each containing 6% (v/v) methanol, but with ion-pairing agent concentration of $0.2 \cdot 10^{-4}$, $0.5 \cdot 10^{-4}$, $1.0 \cdot 10^{-4}$ and $3.0 \cdot 10^{-4}$ *M*. The retention values of all compounds were determined in each solvent and are shown in Fig. 3. From this it can be seen that sodium heptanesulphonate concentrations above $1.2 \cdot 10^{-4}$ *M* give unacceptably high capacity factors for 5HT and its oxidation product. At levels of $0.3 \cdot 10^{-4}$ *M* and below, peaks of adrenochrome, dopamine and oxidised 5HT converge on one another. The best separation of all eight peaks occurs at a sodium heptanesulphonate concentration of $0.45 \cdot 10^{-4}$ *M* and this is shown in Fig. 4.

It would have been possible to achieve a similar separation using gradient rather than isocratic elution. However this would have meant a slower throughput of samples owing to the time taken for the column to re-equilibrate between runs. Also, gradient elution requires more equipment, and therefore greater cost, if reproducible gradients are to be obtained. This study demonstrates that suitable optimisation procedures can be used to produce complex separations, even without the benefit of gradient elution.

Finally, a tube containing a buffered mixture of all four amines, each at a concentration of $1.25 \cdot 10^{-3}$ *M*, was incubated with caeruloplasmin for 45 min at 37°C. Following termination of the reaction with sodium azide, 10 µl of the incubation mixture were injected onto the column and the resultant chromatogram is shown in Fig. 5. This shows that, of the four amines, only adrenaline has undergone oxidation. The observation that adrenaline, or its oxidation product, prevents the oxidation of other biogenic amines by caeruloplasmin may well be of importance in view of the enzymes' postulated rôle as a regulator of amine levels. Further investigations into the oxidation of amine mixtures by caeruloplasmin will be reported elsewhere.

REFERENCES

1 B. C. Barrass and D. B. Coult, *Biochem. Pharmacol.*, 21 (1972) 677.
2 D. A. Richards, *J. Chromatogr.*, 256 (1983) 71.
3 G. Curzon and B. E. Speyer, *Biochem. J.*, 109 (1968) 25.
4 B. C. Barrass and D. B. Coult, *Biochem. Pharmacol.*, 23 (1974) 47.
5 E. C. Juenge, P. E. Flinn and W. B. Furman, *J. Chromatogr.*, 248 (1982) 297.
6 T. P. Moyer, N.-S. Jiang, G. M. Tyce and S. G. Sheps, *Clin. Chem.*, 25 (1979) 256.
7 D. J. Sams, personal communication.