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UNEQUIVOCAL SYNTHESIS AND CHARACTERISATION OF DOPAMINE 3- AND 4-*O*-SULPHATES

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Abstract—The major metabolic products of the endogenous catecholamine dopamine are its 3- and 4-*O*-sulphates which have also been implicated as intermediates in noradrenaline biosynthesis. Because of the unsatisfactory status of the literature concerning the synthesis, isolation, purity and characterisation of the dopamine *O*-sulphates we describe both a one-step synthesis and definitive separation and characterisation procedures for these metabolites. High-performance liquid chromatography (HPLC) combined with high-field nuclear magnetic resonance techniques were employed. The chemical sulphonation of dopamine gave three synthetic products, whose relative amounts depended critically upon the reaction conditions employed. Dopamine 3- and 4-*O*-sulphates together with dopamine 6-sulphonic acid, a hitherto undescribed derivative of dopamine, were for the first time isolated and characterised unequivocally. It should now prove possible to reappraise critically the biological significance of the major metabolite products of dopamine.

Since the discovery of the third endogenous catecholamine dopamine [1–3], several workers have investigated its metabolic transformation by sulphoconjugation. Jenner and Rose [4] were first to demonstrate the *in vitro* conversion of dopamine to its 3- and 4-*O*-sulphates using preparations from rat liver and brain and, as part of this study, they described a one-step synthesis of the authentic metabolites from dopamine and sulphuric acid, a method which has been used extensively hitherto. In this latter study, dopamine hydrochloride was reacted at 0° with a 10-fold molar excess of conc. H_2SO_4 (sp. g. 1.86) to give dopamine 3-*O*-sulphate and dopamine 4-*O*-sulphate (Fig. 1) in 10 and 12% yield respectively. Ion-exchange chromatography and differential crystallisation were used to resolve the two isomers which were characterised by chemical procedures involving methylation and oxidative degradation to isovanillic and vanillic acids. No direct structural analysis was attempted, except for the reaction with Gibbs's reagent, which should give a positive reaction with the 4-*O* isomer but not with the 3-*O* isomer. Subsequent workers [5] modified the reaction conditions, using a 35-fold excess of conc. H_2SO_4 at −12° with a consequent low yield (3%) of the sulphate conjugates. Again, chemical derivatisation was accomplished to characterise the sulphates, but in this case by methylation and oxidation to the corresponding aldehydes isovanillin and vanillin.

From this point, the literature becomes more obscure. Claims that dopamine 3-*O*-sulphate is the major urinary metabolite of orally administered dopamine in the dog [6], that both sulphates can be quantitated in the urine by high-performance liquid chromatography (HPLC) after L-DOPA administration to man [7] and that the sulphates are converted by dopamine β-hydroxylase directly to noradrenaline [8, 9] cannot be evaluated, since none of these workers give any data to substantiate the nature or purity of their synthesised materials.

Like all the aforementioned groups, we have attempted the chemical sulphonation of dopamine as described previously [4], but in our hands the two sulphoconjugates could not be isolated, even after many attempts. This is not totally unexpected, since *O*-sulphates would not be the preferred products of reaction between phenolic derivatives and SO_3 donors such as conc. H_2SO_4. In such cases, sulphonation of the aromatic nucleus, *ortho*/*para* to the phenols, in such a ring system activated towards electrophilic substitution, will be expected to predominate, yielding sulphonic acids [10]. These would be isomers of the *O*-sulphates, indistinguishable from the latter by Gibbs's reaction, i.r. and u.v. spectroscopy and indeed elemental analysis (C, H, N and S).

In view of the biochemical importance which is attached to the dopamine *O*-sulphates, both as metabolic excretory products of dopamine and L-DOPA and as possible intermediates in noradrenaline biosynthesis, unequivocal evidence of the chemical structure of the synthetic products is required. We have thus investigated the original synthetic reaction [4] in more detail, with reference to temp. of reaction and sp. g. of the sulphuric acid employed. In this paper we describe the application of the techniques of HPLC and ^1H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) to the separation, isolation and authentication of the reaction products. Additionally, it will be shown that a major product of this reaction, dependent upon temp. and sp. g. of the H_2SO_4 employed, is not a dopamine

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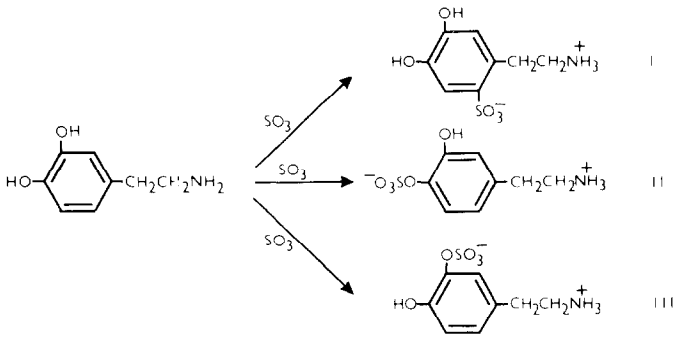

Fig. 1. Products of chemical sulphonation of dopamine. I—dopamine 6-sulphonic acid. II—dopamine 4-*O*-sulphate. III—dopamine 3-*O*-sulphate.

O-sulphate but rather dopamine 6-sulphonic acid [2-(2′-aminoethyl) - 4,5 - dihydroxyphenyl sulphonic acid], a hitherto undescribed compound.

MATERIALS AND METHODS

Materials. Chemicals were obtained from the following sources: dopamine hydrochloride (Sigma Chemical Co., London, U.K.), sulphuric acid (sp. g. 1.92, Hopkin and Williams), pyridine-sulphur trioxide (Aldrich Chemical Co., Gillingham, U.K.), chlorosulphonic acid (BDH Chemicals, Poole, U.K.) and 2,6.dichloro-*p*-benzoquinone-4-chloroimine (Gibbs's reagent, BDH Chemicals, Poole, U.K.). Methanol was of spectroscopic grade and water was glass-distilled and deionised.

Chemical sulphonation of dopamine. The reaction was performed according to Jenner and Rose [4] whereby dopamine hydrochloride (0.4 g, 2.1 mmole) which had been kept over P_2O_5 *in vacuo* for 24 hr was added to stirred conc. H_2SO_4 (sp. g. 1.86; 1.1 ml, 20.6 mmole) at 0°. After 20 min, the mixture was poured into crushed ice–water (10 ml) and the resultant clear solution applied to a Dowex 50X8 ion-exchange column (200–400 mesh, H^+ form; 200 × 15 mm). Elution was performed with water, the effluent monitored at 280 nm and fractions (5 ml) were collected and monitored for pH. All fractions with pH < 3, which contained approx. 70% of the u.v.-absorbing material were pooled. Similarly, fractions with pH ≥ 3 were pooled. According to the published method [4], fractions of pH < 3 should be discarded since they contain largely inorganic sulphate but, because of the quantity of organic material they obviously contained from their high u.v. extinction, it was decided to investigate them further. Both pH < 3 and pH > 3 bulked eluates were treated similarly and reduced to 10 ml *in vacuo* at 30°. Both were frozen and stored as such for 24 hr, then allowed to thaw slowly at 0° as described [4]. Unlike these previously described methods, no crystals appeared in the pH ≥ 3 fraction. However, a copious crop of white crystals were obtained from the more acidic fraction. These were washed with ethanol–ether mixture and dried *in vacuo* over P_2O_5, giving a white crystalline product (67 mg; yield as dopamine sulphates 13.7%), which according to published methods should be the 4-*O*-sulphate. The mother liquor from this fraction was applied to a column of Dowex 1X8, to isolate the 3-*O*-sulphate. Each of 60 10-ml fractions thus collected was retained from high performance liquid chromatography (HPLC) analysis.

Variation of reaction temp. and sp. g. of H_2SO_4. The effect of varying reaction temp. (0–38°) and H_2SO_4 sp. g. (1.84–1.92) was investigated. Reactions were carried out as described above, except that the required sp. g. of H_2SO_4 was obtained by the mixing of fuming H_2SO_4 (sp. g. 1.92) with conc. H_2SO_4 (sp. g. 1.84), using both a hydrometer and density determinations by weighings to adjust the sp. g. At the end of each reaction, the mixture was poured into 10 vol. crushed ice–water mixture which was retained for HPLC analysis. In such a way, reactions were carried out at 0, 20 and 38° using H_2SO_4 of 1.84, 1.86 and 1.92 sp. g.

In addition, sulphonation of dopamine was performed using chlorosulphonic acid according to Rose and Bleszynski (personal communication) and using pyridine-sulphur trioxide [11]. Again, aliquots of diluted reaction mixture were retained for HPLC analysis.

High-performance liquid chromatography (HPLC). For both the analysis and preparative separation of the products of the various sulphonation reactions, HPLC was employed. Diluted reaction mixture (100–200 μl), containing 5–10 mg of dopamine-related material, was injected into a Hypersil ODS (5 μm particle size) column (250 × 8 mm, Shandon Southern Products) and eluted with 2% aq. methanol delivered by a Waters Associates 6000a pump at 0.8–1.0 ml/min. The column effluent was monitored at 284 nm using a Waters variable wavelength detector (Model 450). Four dopamine-related peaks were observed, of variable size depending on reaction conditions, with elution vols. of 9.0, 26.2, 29.2 and 128.8 ml. The last of these co-chromatographed with dopamine under various conditions including in 0.5% aq. methanol. The first three are referred to hereafter as products I, II and III respectively.

Nuclear magnetic resonance spectroscopy (NMR). Products I, II and III (above) were isolated after repetitive injection and reinjection on HPLC and subjected to various analyses including NMR. ^1H NMR spectra were acquired on samples of these (8–15 mg) as solutions in d_6-DMSO (products II and III) or D_2O (product I) using a Bruker NMR spectrometer operating at 250 MHz. Where appropriate,

scale expansion of the spectral region containing the aromatic proton signals (δ6.5–7.5 relative to tetramethylsilane) was made. In addition, natural abundance ^{13}C NMR spectra were recorded for product I (80 mg in D_2O) using another Bruker WM250 spectrometer. Off-resonance-decoupled techniques were used to distinguish between quaternary carbons (ring-substituted carbons) and aromatic carbons bonded to hydrogen.

RESULTS

Sulphonation of dopamine by published methods

The sulphonation of dopamine using a 10-fold excess of cold (0°) conc. H_2SO_4 (sp. g. 1.86) was performed as described previously [4]. Work-up of the reaction mixture using Dowex 50X8 ion-exchange chromatography did not yield a crystalline product from the higher pH (≥3) fractions. Rather, the acid fractions (pH < 3), which it is advised to discard, contained approx. 70% of the u.v.-absorbing material (280 nm) which, when treated as the higher pH fraction, gave a white crystalline product (67 mg; m.p. 244–247 (d)°). Dopamine monosulphate ($C_8H_{11}NO_5S$) requires: C, 41.2; H, 4.7; N, 6.1%. Found: C, 41.1, H, 4.9; N, 6.0%. At first, it was thought that dopamine 4-*O*-sulphate had been isolated, since both chemical yield and crystallisation characteristics were very similar to those reported [4]. However, further examination of this material by HPLC revealed that it was a composite of three substances (products I–III, *vide supra*). This mixture gave a positive Gibbs's reaction and had an acceptable elemental analysis, which had also led us to believe this was the authentic 4-*O*-sulphate. However, the substance was poorly labile to hydrolysis with either sulphatase or 0.1 M HCl, which would not have been expected for an *O*-sulphate. Characterisation by NMR (*vide infra*) of products I–III, which this substance contains, showed that this crystalline product contained both dopamine 3- and 4-*O*-sulphates and a large quantity of a sulphonic acid, the latter neither labile to sulphatase nor 0.1 M HCl.

The mother liquor from the pH < 3 fraction was chromatographed on Dowex 1X8, the method described to isolate the 3-*O*-sulphate [4]. Sixty 10-ml fractions were collected from this column and inspected by HPLC. Fractions 1–25 contained products I and fractions 26–60 contained only dopamine. The identity of product I is given later.

Effect of reaction temp. and H_2SO_4 sp. g. on the products of dopamine sulphonation

Sulphonation of dopamine was performed at 0, 20 and 38° using either 1.84, 1.86 or 1.92 sp. g. H_2SO_4. The approx. content of products I–III and unreacted dopamine was determined in each case by HPLC. The results are given in Table 1. In the case of fuming H_2SO_4 (sp. g. 1.92) at 0° one polar product predominated on HPLC (product I). Small amounts (approx. 5%) of two chromatographically similar products were also observed (products II, III). No unreacted dopamine was found. When this reaction was carried out at 20°, only product I was formed. Reactions using 1.86 sp. g. H_2SO_4 yielded all three products I–III in a temp.-dependent fashion. At 0°, roughly equal amounts of each of these were formed, whilst on raising the temperature to either 20 or 38°, product I again predominated, with only traces of products II and III. With 1.84 sp. g. H_2SO_4 at 0°, approx. half the dopamine remained unreacted, most of the products formed being II and III (20% each), with a little of product I (5%). Reaction of dopamine with chlorosulphonic acid at 20° was found to give mainly unreacted dopamine (70%) with 15% each of products II and III. No reaction could be observed between dopamine and pyridine-sulphur trioxide.

Structural determination of the reaction products by nuclear magnetic resonance spectroscopy (NMR)

Under various chemical conditions, three products (I–III) of the reaction between dopamine and sulphonation agents were observed. Two such products, dopamine 3- and 4-*O*-sulphates have been claimed previously by many workers [4–9]. As discussed previously, sulphonation of dopamine might not only result in the formation of *O*-sulphates, but also one or more sulphonic acids, the ratio between such products being dependent upon the reaction conditions [10]. We therefore isolated 20–100 mg of each of products I, II and III from the various reactions (Table 1) for analysis by NMR techniques. Since both *O*-sulphates and ring sulphonic acids would be isomers, they could only be realistically distinguished by a technique such as NMR which gives discrete information about the aromatic hydrogens (protons) and carbon atoms and their microenvironment.

The results given below demonstrate that products I, II and III are dopamine 6-sulphonic acid, dopamine 4-*O*-sulphate and dopamine 3-*O*-sulphate respectively (Fig. 1).

Table 1. Products of reaction of various sulphonating agents with dopamine at various temps

Reagent	T (°)	Approximate yield of product I (9.0)*	II (26.2)	III (29.2)	Dopamine (128.8)
H_2SO_4 (sp. g. 1.92)	0	90	5	5	n.d.
	20	100	n.d.†	n.d.	n.d.
H_2SO_4 (sp. g. 1.86)	0	30	35	35	n.d.
	20	95	2.5	2.5	n.d.
	38	95	2.5	2.5	n.d.
H_2SO_4 (sp. g. 1.84)	0	5	20	20	55
Chlorosulphonic acid	20	n.d.	15	15	70
Pyridine-sulphur trioxide	20	n.d.	n.d.	n.d.	100

* Elution vol. (ml) in HPLC system (see text).
† n.d. = not detected.

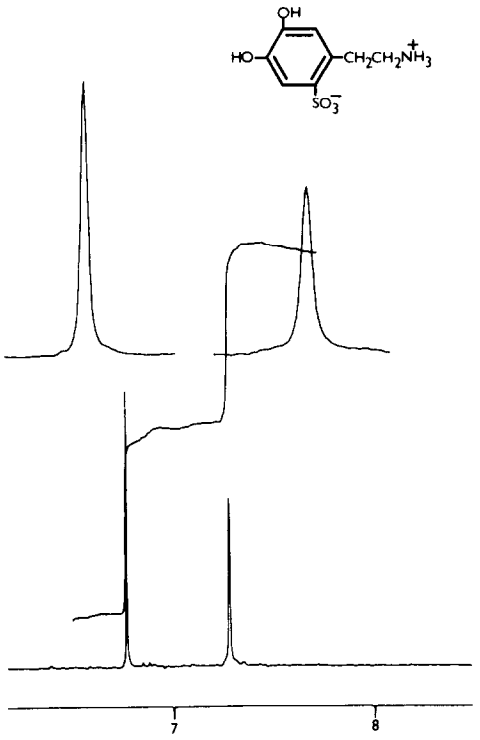

Fig. 2. ^1H NMR spectrum (250 MHz) (aromatic region) or dopamine 6-sulphonic acid (Product I, bottom), including expanded spectrum (top).

Product I

This material was isolated by preparative HPLC (elution vol.: 9 ml in 2% aq. methanol) as described. Dopamine 6-sulphonic acid monohydrate ($C_8H_{11}NO_5S\ H_2O$) requires: C, 38.2; H, 5.2; N, 5.6%. Found: C, 38.5; H, 5.5; N, 5.9%; m.p. 272–275 (d)°. The 250 MHz NMR ^1H spectrum showed signals corresponding to the four aliphatic protons of the dopamine side-chain and two signals down-field each corresponding to the aromatic proton (Fig. 2). Such a configuration could only arise from sulphonation of the aromatic ring, since sulphonation of either phenolic group would give signals arising from three aromatic protons (positions 2, 5 and 6, see Fig. 2). Additionally, since no coupling (splitting) was detectable between these two aromatic protons, even on scale expansion (Fig. 2, top), the protons must be *para* to one another. Interestingly, the lowest frequency signal (2-proton) is broadened, presumably due to a weak through-bond coupling to the aliphatic protons. Thus, the only assignable structure to the empirical formula $C_8H_{11}NO_5S$ is that of the 6-sulphonic acid. This was confirmed by off-resonance-decoupled ^{13}C NMR spectroscopy, which shows four singlets corresponding to four quaternary (substituted) carbons and two doublets (up-field) corresponding to the two carbon-hydrogen bonds (Fig. 3). To the best of our knowledge, dopamine 6-sulphonic acid [2-(2′-aminoethyl)-4,5-dihydroxyphenylsulphonic acid] has not previously been described.

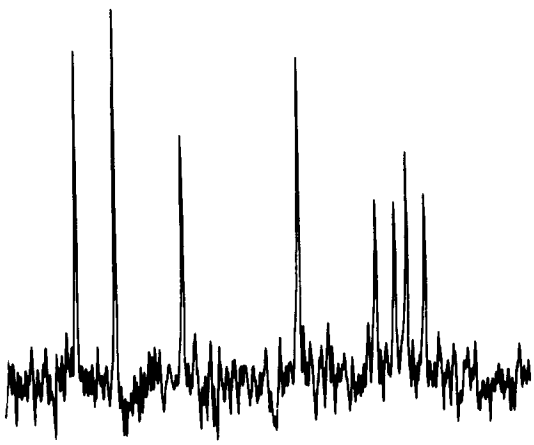

Fig. 3. Off-resonance decoupled ^{13}C NMR spectrum of dopamine 6-sulphonic acid.

Product II

^1H NMR analysis of product II (elution vol.: 26.2 ml in 2% aq. methanol) revealed that the most likely structural assignment is that of dopamine 4-*O*-sulphate. The 250 MHz spectrum showed the four aliphatic protons and three aromatic signals corresponding to each of three protons. The most deshielded proton in this structure (Fig. 4) would be that adjacent to the OSO_3^- grouping, i.e. that in the 5-position. The appearance of this signal as a doublet with a typical *ortho* coupling is consistent with this interpretation. Next up-field, a doublet with a small

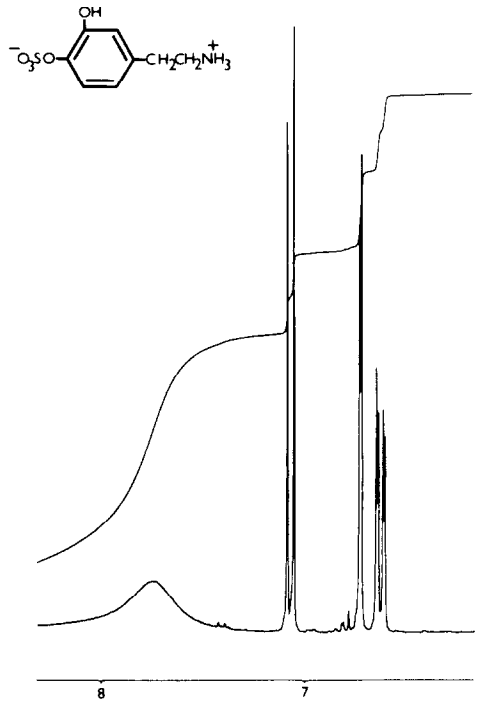

Fig. 4. ^1H NMR (250 MHz) of dopamine 4-*O*-sulphate (Product II).

coupling (*meta*) was observed, which can be assigned to the proton in the 2-position. Finally, a quartet was observed with both *ortho* and *meta* coupling which is indicative of the proton in the 6-position. Such a spectrum could not have been obtained from a ring-substituted sulphonic acid, nor the 3-*O*-sulphate (*vide infra*). Thus, product II is dopamine 4-*O*-sulphate.

Product III

^{1}H NMR analysis of product III (elution vol.: 29.2 ml in aq. methanol) showed that it is undoubtedly dopamine 3-*O*-sulphate (Fig. 5). Apart from the low-frequency signals corresponding to the four aliphatic protons, again three aromatic proton resonances were observed. The highest frequency of these (down-field) was a doublet of small coupling (*meta*) corresponding to a proton adjacent to the OSO_3^- group (2-position). Next was observed a quartet corresponding to the proton in the 6-position which is coupled both *ortho* and *meta*. Finally, the doublet corresponding to the 5-proton, coupled only *ortho*, was observed. Thus, this spectrum could not have arisen from either a sulphonic acid or dopamine 4-*O*-sulphate and therefore product III is dopamine 3-*O*-sulphate.

Inspection of Figs. 4 and 5 also shows that the 3-*O*-sulphate is contaminated with a small quantity (approx. 5%) of the 4-*O*-sulphate, which is consistent with our HPLC purity checks.

DISCUSSION

This study has shown that the published synthesis of dopamine 3- and 4-*O*-sulphates does not yield these two products solely, rather a mixture of dopamine 6-sulphonic acid together with the isomeric sulphates and some unreacted dopamine. It is thus hard to believe that subsequent workers [6–9], who followed the original method [4], could have isolated the pure sulphates. Nevertheless, claims of biological significance, either as metabolic excretory products or intermediates in noradrenaline biosynthesis, have been made for these *O*-sulphates. Of particular note is the notion that noradrenaline can be formed from both dopamine *O*-sulphates in a single step by dopamine β-hydroxylase [8, 9]. In this case, even the Michaelis–Menten kinetics of the reaction were described. This would seem an unwarranted extrapolation without a chemical foundation, since in this paper we have clearly shown that small changes in sp. g. of sulphuric acid or in reaction temp. can give rise to the presence of either dopamine 6-sulphonic acid or unreacted dopamine in the product. Because the three products of dopamine sulphonation are isomeric chemically similar organic acids, they will not be distinguished by elemental analysis, u.v. or i.r. spectroscopy or by Gibbs's reaction, particularly when they are difficult to obtain in a pure form. We considered that NMR was the only technique powerful enough to distinguish these isomers and HPLC the most efficient method of separation and isolation.

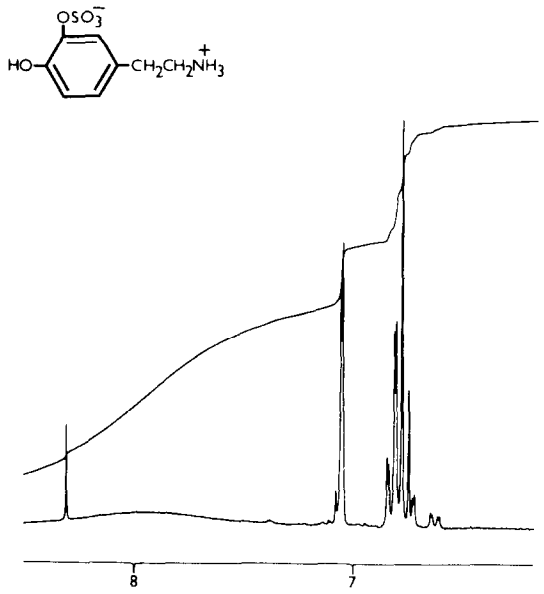

Fig. 5. ^{1}H NMR spectrum (250 MHz) of dopamine 3-*O*-sulphate (Product III).

Because of the formation of dopamine 6-sulphonic acid, we would strongly advocate the use of HPLC both to monitor the purity of synthesised dopamine sulphates and to separate mg quantities of these for biochemical experiments. It is possible that the 6-sulphonic acid, 4-*O*-sulphate and 3-*O*-sulphate could be identified from their retention vols. (2% aq. methanol) on reversed-phase HPLC (5 μm Hypersil ODS) relative to the retention vols. of dopamine itself. Relative retention vols., calculated from data in Table 1 are 0.07, 0.20 and 0.23 respectively for the above derivatives.

In recent years, the third endogenous catecholamine dopamine has assumed greater importance due to the recognition of its physiological effects mediated through a specific receptor in the CNS, cardiovascular system and the kidney [12]. Furthermore, the endogenous precursor of dopamine, L-DOPA, is widely used in the treatment of Parkinsonism [13, 14]. Obviously, the metabolic transformation of the neurohormone and its precursor may be important factors in the physiological regulation of dopaminergic effects. If conjugation with sulphate is a major component of dopamine metabolism as suggested [4, 6, 7, 15], it is important to quantitate the sulphates using authenticated synthetic standards. In this paper, we describe for the first time a one-step unequivocal synthesis and separation of the authentic dopamine sulphates to >95% purity using HPLC and NMR. In theory, material isolated from a single preparative HPLC run, whether it be from chemical or biological origin, should be sufficient for definitive structural assignment by modern 250 or 400 MHz NMR techniques, and thus we would wish to underline the usefulness of these two combined analytical techniques in the small-scale isolation and characterisation of, for example, the dopamine sulphoconjugates. Additionally, this paper provides the basis for a reappraisal of the biological importance of the dopamine 3- and 4-*O*-sulphates.

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