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DETERMINATION OF DOPAMINE-O-SULFATE AND NOREPINEPHRINE-O-SULFATE ISOMERS AND SEROTONIN-O-SULFATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING DUAL-ELECTRODE ELECTROCHEMICAL DETECTION

MARY ANN ELCHISAK
Department of Veterinary Physiology and Pharmacology, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907 (U.S.A.)
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SUMMARY

This report describes the use of high-performance liquid chromatography with dual series electrode electrochemical detection to quantitate dopamine (DA)-3-O-sulfate and DA-4-O-sulfate, as well as norepinephrine (NE)-O-sulfate isomers and serotonin (5-HT)-O-sulfate. An oxidation potential was maintained at the upstream (W1) electrode, and a reduction potential at the downstream (W2) electrode. For DA- and NE-sulfates, the sulfate moiety, hydrogen and electrons are most likely removed at W1 and the corresponding quinones formed. At W2, the electrons are recaptured, creating a reductive current (peak). Results indicate that this is a sensitive procedure for the determination of DA- and NE-sulfate isomers. It is less complex than a recently developed post-column hydrolysis procedure, and is at least equivalent to that procedure in terms of specificity and lower limits of detection (< 1 picomole) for the DA- and NE-sulfate isomers. The procedure is relatively insensitive for 5-HT-O-sulfate, as well as for tyramine-O-sulfate, homovanillic acid-sulfate and 3-methoxy-4-hydroxyphenyl glycol sulfate. It should prove useful for investigations concerning the specific conjugates of DA or NE in body tissues and fluids.

INTRODUCTION

Investigations concerning the specific conjugates of dopamine (DA) in the body have been hampered by the lack of specific and sensitive methodology for their detection. Most available methods do not distinguish between the two isomers of DA-sulfate, DA-3-O-sulfate and DA-4-O-sulfate, even though both isomers have been reported to occur after 1-DOPA administration in urine from humans[1–3] and pigtail monkey[4].

This laboratory has recently developed a high-performance liquid chromatographic (HPLC) method utilizing ultraviolet detection at 280 nm to separate and quantitate each of the DA-sulfate isomers[5]. However, the lack of sensitivity and specificity of the ultraviolet detection method precluded use of this technique for

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routine analysis of DA-3-O-sulfate and DA-4-O-sulfate in biological tissues or fluids. Although DA-3-O-sulfate and DA-4-O-sulfate could be detected by single-electrode electrochemical detection, this technique was also unsuitable for trace determinations of these compounds because of the requirement for an oxidation potential in excess of +1.2 V (ref. 6). The high oxidation potential resulted in excessive baseline fluctuations and noise due to oxidation of components of the mobile phase and sample matrix, as well as short electrode life. A more sensitive technique for this purpose utilizing post-column hydrolysis of each DA-sulfate isomer to free DA coupled with single-electrode electrochemical detection of the free DA at a relatively low oxidation potential was then developed[6]. The present report describes the use of HPLC with dual electrode electrochemical detection to quantitate the DA-sulfate isomers. The dual-electrode technique reported here is less complicated than the post-column hydrolysis procedure, and is at least equivalent to that technique in terms of specificity and lower limits of detection for the DA-sulfate isomers.

The dual-electrode technique was also found to be suitable for specific and sensitive detection of norepinephrine (NE)-3-O-sulfate and NE-4-O-sulfate. The technique was relatively insensitive for determination of homovanillic acid sulfate (HVA-sulfate; 3-methoxy-4-[hydroxysulfonyloxy]phenylacetic acid); 3-methoxy-4-hydroxyphenyl glycol sulfate (MHPG-sulfate; 3-methoxy-4-[sulfonyloxyphenyl]-glycol), serotonin-O-sulfate (5-HT-sulfate; 5-hydroxytryptamine-O-sulfate) and tyramine-O-sulfate.

METHODS

Chromatographic apparatus

The liquid chromatograph consisted of the following components, purchased from Bioanalytical Systems (West Lafayette, IN, U.S.A.) or Waters Assoc. (Milford, MA, U.S.A.): a Waters Model 6000-A pump, a Rheodyne Model 7125 injection valve equipped with a 20-μl loop and a Houston Instruments dual-channel B-5000 strip-chart recorder. A column of air connected in parallel with the solvent flow-path served as a pulse damper. The system was equipped with a stainless-steel column (25 cm × 4 mm I.D.) prepacked with 5-μm Biophase octadecyl silica (Bioanalytical Systems) and a guard column (2 cm × 4.5 mm I.D.) packed with 40 μm C_{18}/Corasil (Waters Assoc.). The detector was an LC-4B/17(D) amperometric detector for liquid chromatography with dual electrode (Bioanalytical Systems). The detector consisted of a thin-layer dual glassy-carbon electrode in the series configuration, an Ag/AgCl reference electrode mounted downstream from the dual electrode, and two LC-4B amperometric controllers for electrochemical detection.

Chromatographic conditions

Chromatographic separation of the sulfated compounds was accomplished at ambient temperature by reversed-phase paired-ion chromatography. The conditions have previously been described in detail for DA-3-O-sulfate and DA-4-O-sulfate[5]. The column was octadecylsilica and the mobile phase was monochloroacetic acid (25 m*M*), pH 2.8, containing ethylenediaminetetraacetate (EDTA) (1 m*M*) and *n*-octylamine (4 m*M*). The mobile phase was passed through a 0.45-μm filter, degassed by sonication, maintained at approximately 63°C, and stirred continuously during use.

The mobile phase flow-rate was usually 0.8 ml/min. The pressure was approximately 1800 p.s.i.

Detector conditions

The thin-layer glassy-carbon dual electrodes were utilized in the series configuration. Potentials were maintained *versus* an Ag/AgCl reference electrode located downstream. For many experiments, the potential was +1.00 V on the upstream (W1) electrode and 0.00 V on the downstream (W2) electrode. The potential was maintained, and varied as indicated in some experiments, with LC-4B amperometric controllers.

Sulfated compounds

The following sulfated compounds were furnished by Drs. Albert A. Manian and J. Stephen Kennedy, Neurosciences Research Branch, National Institute of Mental Health (Rockville, MD, U.S.A.); DA-3-O-sulfate, DA-4-O-sulfate, NE-3-O-sulfate, NE-4-O-sulfate, 5-HT-sulfate, tyramine-O-sulfate and HVA-sulfate. MHPG-sulfate was obtained from Fluka (Buchs, Switzerland). For some experiments, DA-3-O-sulfate and DA-4-O-sulfate were synthesized and purified according to a modification of the method of Jenner and Rose[7] described previously[5].

RESULTS AND DISCUSSION

Capacity factors

Each of the compounds of interest eluted from the HPLC column in a clearly defined peak. Capacity factors are given in Table I. None of the compounds injected interfered with the detection of any of the other compounds, except that NE-3-O-sulfate and NE-4-O-sulfate were not satisfactorily resolved from each other under the conditions utilized. Consequently, the chromatographic conditions would have to be changed if one wishes to distinguish between NE-3-O-sulfate and NE-4-O-sulfate in samples in which a mixture of the two might occur. Since the primary interest of this laboratory concerns DA-3-O-sulfate and DA-4-O-sulfate, no attempt was made to optimize the separation of NE-3-O-sulfate and NE-4-O-sulfate.

TABLE I

RETENTION TIMES EXPRESSED AS CAPACITY FACTORS

Capacity factor, k' = (peak retention time − void volume time)/void volume time.

Compound	*Capacity factor*
Norepinephrine-3-O-sulfate	0.80
Norepinephrine-4-O-sulfate	0.90
Tyramine-sulfate	1.87
3-Methoxy-4-(hydroxysulfonyloxy)phenylacetic acid (HVA-sulfate)	3.00
Dopamine-3-O-sulfate	3.27
Dopamine-4-O-sulfate	3.67
3-Methoxy-4-hydroxy phenylglycol sulfate	7.40
Serotonin-O-sulfate	10.80

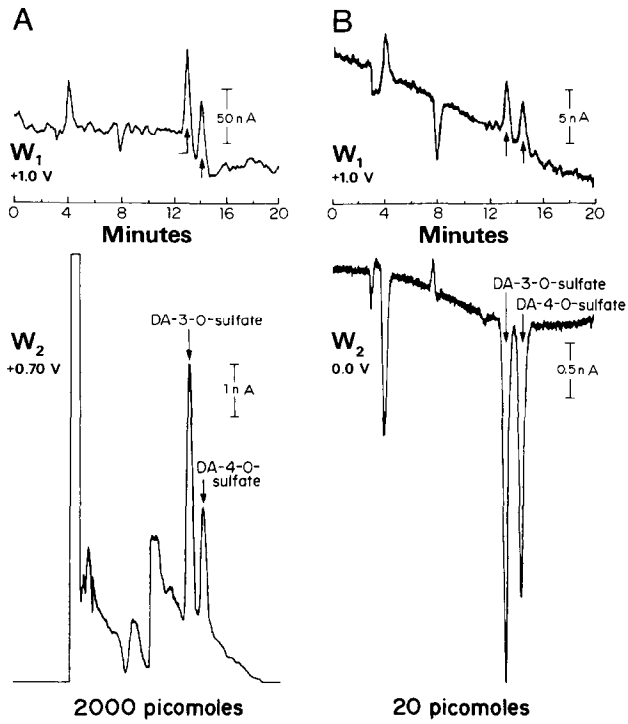

Fig. 1. Series dual-electrode chromatograms for DA-3-O-sulfate and DA-4-O-sulfate. Injection volume was 20 μl. Other HPLC conditions were as described in the text. (A) 2000 pmoles of each DA-sulfate isomer was injected. Both electrodes were maintained at oxidation potentials, W1 = +1.00 V and W2 = +0.70 V. (B) 20 pmoles of each DA-sulfate isomer was injected. Upstream electrode was maintained as in (A), downstream electrode (W2) was maintained at reduction potential of 0.00 V.

Determination of DA-sulfate isomers

The series chromatograms for DA-3-O-sulfate and DA-4-O-sulfate, with the upstream (W1) potential maintained at +1.00 V and the downstream electrode (W2) at +0.70 V, are shown in Fig. 1A. A 2000-pmole (2-nmole) sample of each isomer was injected. The direction of the DA-sulfate peaks indicates oxidative responses (loss of electrons) at both electrodes. Quantitation of the upstream response is limited by the oxidation of mobile phase components at the relatively high potential necessary for the oxidation of the DA-sulfate isomers. This response is essentially identical to that which occurs when DA-sulfate isomers are detected by a single electrode electrochemical detector. Direct detection of small amounts of DA-3-O-sulfate and DA-4-O-sulfate by single electrode electrochemical detection is usually unsuitable for routine use because the hydrodynamic voltammogram of each isomer indicates that maximal oxidation of these compounds requires an oxidation potential in excess of 1.2 V (ref. 6). Experiments described later in this paper (Fig. 2) suggest that an oxidation potential of +1.4 V does not produce maximal oxidation of the DA-sulfate isomers. Very high oxidation potentials such as these usually result in excessive baseline fluctuations as well as a relatively short-lived electrode surface.

Fig. 1A also shows that a response to DA-sulfate isomers was also obtained at

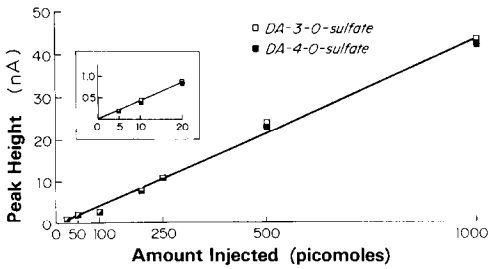

Fig. 2. Standard curve for DA-3-O-sulfate and DA-4-O-sulfate determined with series dual-electrode detection. Responses were measured at W2. W1 = +1.00 V, W2 = 0.00 V. HPLC conditions were as described in the text.

the downstream (W2) electrode maintained at +0.70 V. However, the response is very small for the amount (2000 pmoles) of each isomer injected. Free DA is easily oxidized at a potential of +0.70 V, while DA-sulfate isomers are not readily oxidized at such a low potential[6]. It was initially thought that free DA formed from DA-sulfate at W1 might be detected at W2. However, the peak heights of DA-3-O-sulfate and DA-4-O-sulfate were not markedly changed when W2 was held constant at +0.70 V and W1 was varied between 0.00 V and +1.10 V (data not shown). It therefore seems unlikely that the response at the downstream electrode is due to free DA formed at W1. The response at W2 could simply be due to oxidation of a very small proportion of the DA-sulfate injected, since the relatively large quantity injected was probably not substantially depleted at the upstream electrode. This could not be tested experimentally by injection of smaller amounts of DA-sulfate because of the instability of the W2 baseline at positive oxidation potentials.

It is apparent from Fig. 1A and the discussion above that use of the dual series electrode at two positive oxidation potentials is not suitable for detection of picomolar amounts of DA-3-O-sulfate or DA-4-O-sulfate.

Fig. 1B shows the series chromatograms for DA-3-O-sulfate and DA-4-O-sulfate, with the upstream (W1) potential maintained at +1.00 V and the downstream electrode (W2) at +0.00 V. The sample size of each isomer injected was 20 pmoles. The direction of the DA-sulfate peaks indicates an oxidative response (loss of electrons) at W1, and a reductive response (gain of electrons) at W2. Quantitation of the upstream response is limited by the oxidation of mobile phase components at the high oxidation potential. This was discussed above for Fig. 1A. A large reductive response was produced at W2 by injection of 20 pmoles of each DA-sulfate isomer. Measurement of the response at W2 under these conditions appears to be more selective than measurement at W1, since there are fewer peaks present. Furthermore, after the electrode has stabilized, there is little or no fluctuation of the baseline at W2. The electrode had not yet completely stabilized in Fig. 1B. The exact mechanism of the reactions at each electrode have not been determined, but it seems likely that oxidation of DA-sulfate at W1 produces a quinone, just as the oxidation of free DA produces a quinone. By setting W2 at an appropriate reduction potential (0.00 V), the quinone is reduced by "recapture" of electrons. It appears from Fig. 1B that low picomolar amounts of DA-3-O-sulfate and DA-4-O-sulfate can be detected at the downstream electrode of a dual series electrochemical detector. The upstream elec-

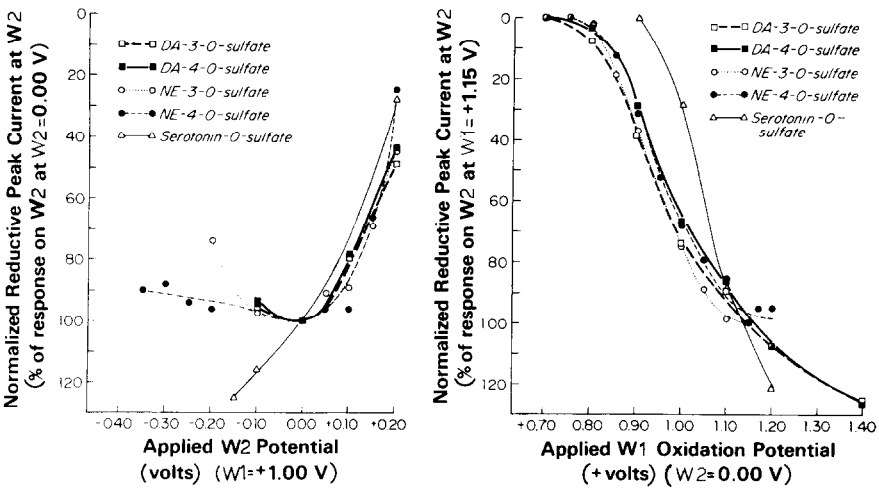

Fig. 3. Hydrodynamic voltammograms of DA-3-O-sulfate (□), DA-4-O-sulfate (■), NE-3-O-sulfate (○), NE-4-O-sulfate (●), and 5-HT-sulfate (△) determined with series dual-electrode detection. A solution containing 200 pmoles of each compound in a volume of 20 μl was injected. HPLC conditions were as described in the text. (A) W1 was held constant at +1.00 V, and W2 varied from −0.35 V to +0.20 V. Responses were measured at W2 and expressed as a percentage of the appropriate response at W2 = 0.00 V. (B) W2 was held constant at 0.00 V, and W1 varied from +0.70 V to +1.40 V. Responses were measured at W2 and expressed as a percentage of the response at W1 = +1.15 V.

trode should be maintained at a suitable oxidation potential, and the downstream electrode maintained at a suitable reduction potential. A similar oxidation–reduction coupling has recently been utilized for the dual electrode detection of the catechol, caffeic acid[8].

Linearity of DA-sulfate response

Injection of varying amounts of DA-3-O-sulfate and DA-4-O-sulfate between 5 and 1000 pmoles indicated that there was a linear correlation between the downstream detector response and the amount of each DA-sulfate isomer injected (Fig. 2). The response was not linear above 1000 pmoles. The upstream electrode was maintained at +1.00 V, and the downstream electrode was maintained at 0.00 V. Consequently, this method could be used to quantitate either or both of the DA-sulfate isomers in biological tissues or fluids.

Optimal electrochemical conditions

Hydrodynamic voltammograms for DA-3-O-sulfate, DA-4-O-sulfate, NE-3-O-sulfate, NE-4-O-sulfate and 5-HT-sulfate are shown in Fig. 3. Fig. 3A shows the responses measured at W2, while W1 was held constant at +1.00 V and W2 was varied from −0.35 V to +0.20 V. The voltammogram indicates that the optimal potential (that producing the maximum response) for W2 is at or very near 0.00 V for each of the DA- or NE-sulfate isomers. For 5-HT-sulfate, however, a W2 potential more negative than −0.20 V is necessary to produce a maximal response (all data not shown). Fig. 3B shows the responses measured at W2. In this case, W2 was held constant at 0.00 V and W1 was varied from +0.70 V to +1.40 V. The results indicate

TABLE II

LOWER DETECTION LIMITS AND OPTIMAL DUAL-ELECTRODE POTENTIALS FOR DETERMINATION OF SULFATED AMINES

Compound	*Signal-to-noise (20 pmoles injected)**	*Lower limits of detection (pmoles)***	*Optimal potential (V)*	
			W1	*W2*
Dopamine-3-O-sulfate	45	0.9	> +1.40	0.00
Dopamine-4-O-sulfate	34	1.2	> +1.40	0.00
Norepinephrine-3-O-sulfate	60	0.7	+1.15	0.00
Norepinephrine-4-O-sulfate	49	0.8	+1.15	0.00
Serotonin-O-sulfate	3	13	> +1.20	< −0.20

* W1 = +1.00 V; W2 = 0.00 V.

** Injected amount producing signal-to-noise ratio of approximately 2; W1 = +1.00 V, W2 = 0.00 V.

that an oxidation potential (applied at W1) in excess of +1.40 V is necessary to produce maximal oxidation of the DA-sulfate isomers, while a potential of approximately +1.15 V appears to be optimal for the oxidation of the NE-sulfate isomers. A potential greater than +1.20 V is apparently necessary for maximal oxidation of 5-HT-sulfate.

The optimal oxidation (W1) and reduction (W2) potentials necessary for the detection of each of the sulfated amines detailed above are summarized in Table II. These results are based on the hydrodynamic voltammograms shown in Fig. 3.

Lower limits of detection

Table II shows the signal-to-noise ratios for 20 picomoles of each of the DA-, NE- and 5-HT-sulfates. W1 was maintained at +1.00 V and W2 was maintained at 0.00 V, even though these potentials were not necessarily optimal for each compound. The approximate lower limit of detection is defined as the amount of a given compound injected which produced a signal-to-noise response ratio at W2 of approximately 2. With W1 at +1.00 V and W2 at 0.00 V, the lower limit of detection ranged from 0.7 to 1.2 pmoles per injection for each of the DA- and NE-sulfate isomers, while 5-HT-sulfate required a 13-pmole injection to produce the same response.

The dual-electrode detection system, under the conditions utilized in the present study (W1 = 1.00 V, W2 = 0.00 V), was less satisfactory than other detection systems for analysis of 5-HT-sulfate. The lower limit of detection for this compound utilizing ultraviolet detection at 280 nm was approximately 11 pmoles[9]. A post-column hydrolysis procedure, described previously[6], resulted in lower detection limits for 5-HT-sulfate of approximately 6 pmoles[9].

The actual lower detection limits could be substantially increased for most of these compounds by increasing the oxidation potential at W1. While this does not necessarily increase the noise generated at W2, an increased W1 oxidation potential usually requires more frequent polishing of the electrode surface. This procedure requires only a few minutes, however, plus an equilibration time which varies depending on the mobile phase composition.

Other sulfate compounds

The dual electrode system was also utilized in an attempt to detect each of the following compounds: HVA-sulfate, MHPG-sulfate and tyramine-O-sulfate. The dual electrode detection system, under the conditions utilized in the present study (W1 = 1.00 V, W2 = 0.00 V), was less satisfactory than other detection systems for analysis of any of these compounds. Peaks were not reliably produced at W2 for an injection of 200 pmoles of MHPG-sulfate or HVA-sulfate. A lower detection limit of 25 pmoles was obtained for tyramine-O-sulfate. When W1 was increased to 1.20 V, the lower detection limit for tyramine-O-sulfate was 8 pmoles. Ultraviolet detection at 280 nm was suitable for each of these compounds, although there are often problems with specificity with this detection mode. Lower limits of detection utilizing ultraviolet detection were 18, 6 and 9 picomoles, respectively, for HVA-sulfate, MHPG-sulfate and tyramine-O-sulfate[9].

Example of use

Injection of human urine (20 μl) directly onto the HPLC column produced a peak at the W2 electrode which co-chromatographed exactly with DA-3-O-sulfate (Fig. 4A and B). Quantitation of the amount of DA-3-O-sulfate represented by this peak, and calculation of a 24-h urinary excretion rate for DA-3-O-sulfate, produced a value of 1.40 μmoles/day in this subject. This value is within the range previously

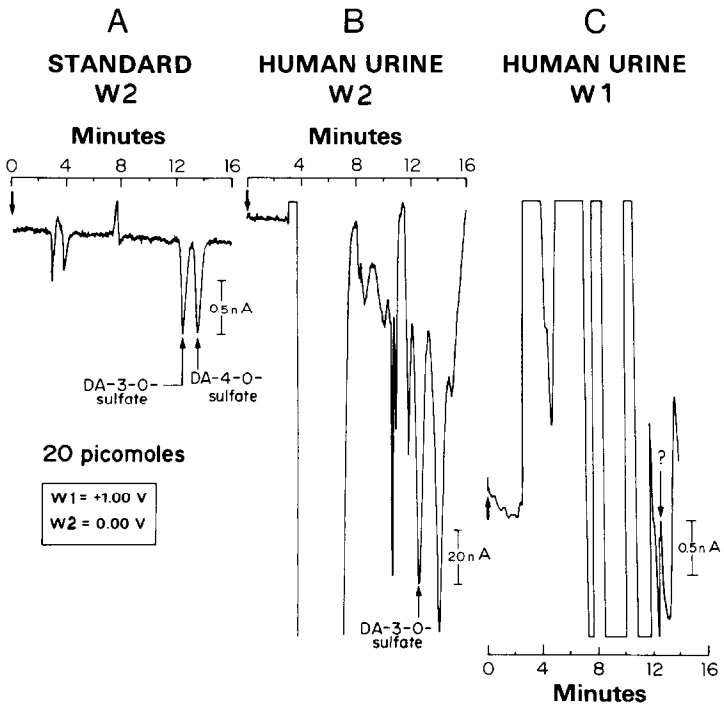

Fig. 4. Series dual-electrode chromatograms for 20 μl of human urine injected directly onto the HPLC column. W1 = +1.00 V, W2 = 0.00 V. Other conditions were as described in the text. (A) Standard solution containing 20 picomoles of each DA-sulfate isomer. Responses monitored at W2. (B, C) Human urine sample injected and responses monitored at W2 (B) and W1 (C).

reported by this laboratory using a much more tedious method[5]. There were two peaks roughly corresponding to DA-3-O-sulfate when the urine was monitored at the W1 electrode (Fig. 4C). If the appropriate peak were identified, quantitation would be very difficult, if not impossible, because of the very large responses seen at W1 and the accompanying difficulty of finding appropriate minima from which to measure the peak.

CONCLUSIONS

This report has described a very sensitive dual electrode electrochemical detection technique for the determination of DA-3-O-sulfate, DA-4-O-sulfate, NE-3-O-sulfate and NE-4-O-sulfate. Lower limits of detection for each of these compounds were found to be approximately 1 pmole under the conditions utilized, which were not necessarily optimal conditions. The technique was more sensitive for each of these compounds than either ultraviolet detection at 280 nm or a post-column hydrolysis technique utilizing single electrode electrochemical detection. For serotonin-O-sulfate, dual-electrode electrochemical detection was less sensitive than either ultraviolet detection or the post-column hydrolysis technique. However, the conditions utilized were not optimal for the detection of serotonin-O-sulfate. Maintenance of a significant reduction potential (< -0.20 V) on W2 appears to be necessary for maximal detection of this compound, and this was not pursued in depth in the present study.

The dual-electrode system was also utilized in an attempt to detect each of the following compounds: HVA-sulfate, MHPG-sulfate and tyramine-O-sulfate. The dual-electrode detection system, under the conditions utilized in the present study (W1 = 1.00 V, W2 = 0.00 V), was less satisfactory in terms of lower detection limits than other detection systems for analysis of any of these compounds. No attempts were made to determine optimal detection conditions for these three compounds. It must be realized that the dual-electrode technique might offer advantages, such as increased specificity, which are not related to the lower limits of detection.

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