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DETERMINATION OF CONJUGATED COMPOUNDS BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION USING POST-COLUMN HYDROLYSIS

APPLICATION TO DOPAMINE SULFATE ISOMERS

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SUMMARY

This report describes a post-column hydrolysis technique for the determination of dopamine-3-O-sulfate and dopamine-4-O-sulfate. The dopamine-sulfate isomers are separated by reversed-phase ion-pair liquid chromatography. Dopamine-3-O-sulfate and dopamine-4-O-sulfate are each hydrolyzed to free dopamine shortly after elution from the liquid chromatography column. Each isomer is then detected as free dopamine by electrochemical detection. Use of the technique results in a fifteen-fold increase in the signal-to-noise ratio for each of the dopamine-sulfate isomers when compared to a previous detection method utilizing ultraviolet detection.

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. Both isomers occur endogenously in urine from Rhesus monkeys[1] and after L-DOPA administration in pigtail monkey[2] and human urine[3–5]. This laboratory has recently developed a high-performance liquid chromatography (HPLC) method utilizing ultraviolet detection to separate and quantitate each of the DA-sulfate isomers[6]. This communication reports an improved technique for this purpose utilizing post-column hydrolysis coupled with electrochemical detection. A specific procedure to hydrolyze each DA-sulfate isomer to free DA as it is eluted from the HPLC column and then to detect it as free DA utilizing electrochemical detection is described. Conditions for optimal hydrolysis are detailed. Use of the technique results in a fifteen-fold increase in the signal-to-noise ratio for each of the two DA-sulfate isomers. The technique is applicable to the analysis of any compound which undergoes acid or base hydrolysis to produce electrochemically active species.

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METHODS

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[6].

General principle and description

DA-3-O-sulfate and DA-4-O-sulfate are separated by HPLC and detected by an ultraviolet (UV) detector at 280 nm. After passing through the UV detector, the column effluent flows into a mixing chamber into which a strong acid is continuously introduced. The effluent exits the mixing chamber and flows through a coil maintained at a high temperature. DA-3-O-sulfate and DA-4-O-sulfate are each hydrolyzed to free DA in the heated coil. The effluent is then passed through a cooling chamber and into an electrochemical detector. Free DA is detected as each of two separate peaks corresponding to each of the original DA-sulfate isomers.

Chromatographic apparatus

The basic liquid chromatograph consisted of the following components purchased from Bioanalytical Systems (West Lafayette, IN, U.S.A.): a Waters M-45 pump, a Rheodyne Model 7125 injection valve equipped with a 20-μl or 200-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 octadecyl silica (Bioanalytical Systems) and a guard column (2 cm × 4.5 mm I.D.) packed with 40 μm C_{18}/Corasil (Waters Assoc., Milford, MA, U.S.A.). Two detectors were utilized: a Waters Model 440 UV absorbance detector equipped with a 280 nm filter and an electrochemical detector (Bioanalytical Systems) consisting of a thin-layer glassy carbon working electrode, an Ag/AgCl reference electrode, and an LC-4 or LC-4A controller. Only an electrochemical detector is required for routine use. Use of the less specific UV detection mode was necessary only for the initial methods development.

Post-column hydrolysis apparatus

The basic liquid chromatography system was modified for the post-column hydrolysis procedure from commercially available parts. The post-column hydrolysis apparatus was connected in series between the UV and electrochemical detectors. Stainless-steel tubing (0.01 in. I.D.) was used throughout the system, except between the cooling chamber exit and the working electrode of the electrochemical detector. These components were connected with polyethylene tubing (0.034 in. I.D.). The

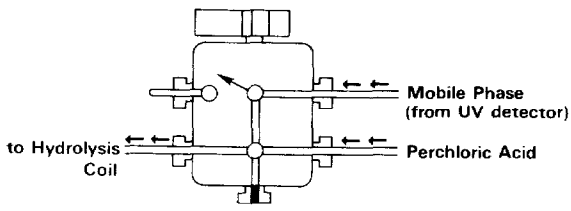

Fig. 1. Schematic diagram of mixing chamber showing mobile phase and perchloric acid flow directions. The flow paths shown resulted in optimal mixing of the acid and mobile phase.

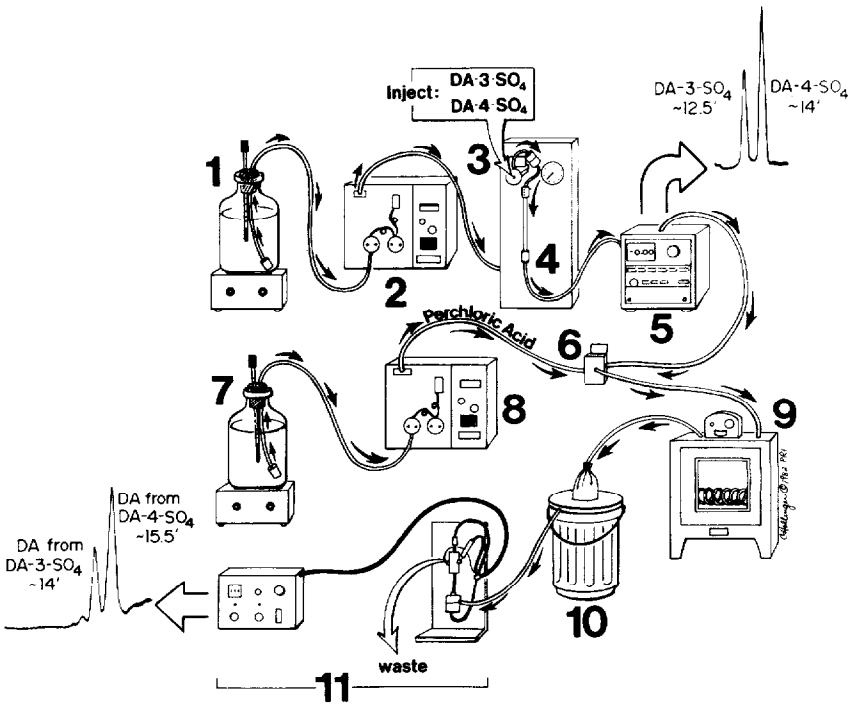

Fig. 2. Schematic diagram of the liquid chromatography system, including the post-column hydrolysis apparatus. Details are given in text. 1 = Mobile phase; 2 = pump; 3 = injector; 4 = HPLC column; 5 = UV detector; 6 = mixing chamber; 7 = perchloric acid; 8 = pump; 9 = hydrolysis oven; 10 = cooling bath; 11 = electrochemical detector.

mixing chamber was constructed from a reference manifold assembly for a Model 6000A pump (Waters Assoc., part No. 25747) (Fig. 1). This was connected to the outlet port of the UV detector. The acid used for the hydrolysis procedure was pumped into the mixing chamber by either a Waters Model M-45 pump or a Constametric I pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.). A column of air connected in parallel with the acid flow served as a pulse damper. The effluent from the mixing chamber then passed through a stainless-steel coil maintained at a constant high temperature in an electric oven. The hydrolysis of DA-3-O-sulfate and DA-4-O-sulfate to free DA occurs during passage through this heated coil.

The hydrolysis coil was connected to a cooling coil which was immersed in a Dewar flask containing an ice–water mixture. The effluent then flowed into the electrochemical detector, where the hydrolysis products (and any other electrochemically active species) were detected.

A diagram of the entire liquid chromatography system, including the post-column hydrolysis apparatus, is shown in Fig. 2.

Chromatographic conditions

The separation of DA-3-O-sulfate and DA-4-O-sulfate was accomplished at ambient temperature by reversed-phase ion-pair chromatography. The conditions have previously been described in detail by Elchisak and Carlson[6]. The column was

octadecyl silica, and the mobile phase was monochloroacetic acid (25 m*M*), pH 2.8, containing 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 0.6 ml/min for routine use. This generated a pressure of approximately 1000 p.s.i. UV detection at 280 nm was utilized. Typical capacity ratios for DA-3-O-sulfate and DA-4-O-sulfate were 2.40 and 2.75, respectively, in this system. The selectivity factor was 1.15.

Post-column hydrolysis conditions

Conditions were as detailed below unless otherwise noted. Perchloric acid (0.4 *M*) was used for the hydrolysis reaction. The acid was passed through a 0.45-μm filter, degassed by sonication, maintained at approximately 63°C and stirred continuously during use. The acid was pumped into the mixing chamber at a rate equal to the mobile phase flow-rate (0.6 ml/min). Consequently, the perchloric acid concentration in the hydrolysis coil was 0.2 *M*. The hydrolysis coil was constructed from stainless-steel tubing (50 ft. × 0.01 in. I.D.). The coil was passed through a standard laboratory oven maintained at 145°C. An elapsed time of 1 min was used for the hydrolysis reaction. The hydrolysis coil was connected to a cooling coil which was immersed in a Dewar flask containing an ice–water mixture. After exit from the cooling coil, the streamline, containing free DA formed from the DA-sulfate isomers, was then passed into an electrochemical detector. A thin-layer glassy-carbon working electrode with the potential maintained at +0.70 V *versus* an Ag/AgCl reference electrode was used.

Boiling of the mixture during the hydrolysis reaction was prevented by maintaining approximately 200 p.s.i. of backpressure in the hydrolysis coil. This was accomplished by placing a constriction valve on the polyethylene tubing between the cooling coil and the working electrode of the electrochemical detector. Maintainance of both the mobile phase and perchloric acid reservoirs at elevated temperature was necessary to completely eliminate the release of dissolved gases during the hydrolysis procedure.

RESULTS AND DISCUSSION

Determination of DA-sulfate isomers pre- and post-hydrolysis

Chromatograms of DA-3-O-sulfate and DA-4-O-sulfate standards before and after the post-column hydrolysis procedure are shown in Fig. 3. Approximately 1.5 nmoles of DA-3-O-sulfate and 2.5 nmoles of DA-4-O-sulfate were injected into the HPLC system. Conditions were as detailed in the methods section. The pre-hydrolysis peaks correspond to DA-3-O-sulfate or DA-4-O-sulfate determined directly by UV detection, and the post-hydrolysis peaks correspond to the free DA formed from each of the DA-sulfate isomers determined by electrochemical detection. The heights of the peaks corresponding to each DA-sulfate isomer were increased approximately fifteen-fold after the post-column hydrolysis procedure (Fig. 3A). When the hydrolysis coil was maintained at room temperature, rather than at 145°C, no hydrolysis of either DA-sulfate isomer to free DA occurred (Fig. 3B). Similarly, no hydrolysis occurred when water was substituted for perchloric acid in the heated hydrolysis coil (data not shown).

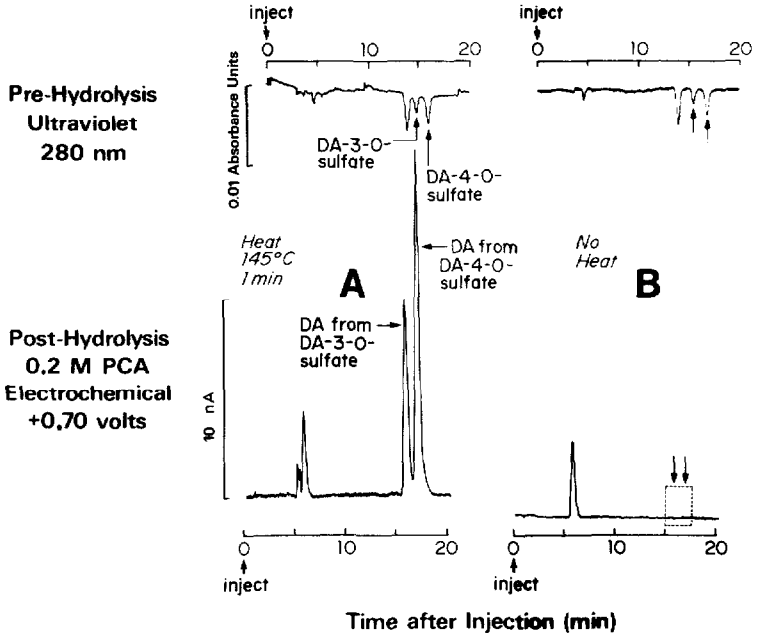

Fig. 3. (A) Chromatograms of DA-sulfate isomers before and after the post-column hydrolysis procedure. The pre-hydrolysis peaks are DA-3-O-sulfate and DA-4-O-sulfate determined by UV detection at 280 nm. The free DA peaks on the post-hydrolysis chromatogram correspond to each of the original DA-sulfate isomers, and were detected electrochemically. Approximately 1.5 nmoles of DA-3-O-sulfate and 2.5 nmoles of DA-4-O-sulfate were injected onto the column. Conditions are detailed in text. (B) The same sample as in (A) except that the hydrolysis coil was at room temperature.

Determination of optimal conditions

The purpose of this series of experiments was to determine the effects of different acids, acid concentration, hydrolysis temperature, and heating time on the recovery of free DA from DA-3-O-sulfate and DA-4-O-sulfate after the post-column hydrolysis procedure. Conditions were as detailed in the methods section, unless the particular parameter was being varied. Hydrolysis of the DA-sulfate isomers with perchloric acid produced larger post-hydrolysis free DA peaks than any of the other acids tested (sulfuric, acetic, and phosphoric) at final concentrations of 0.5 and 0.33 *M* (data not shown). The height of the post-hydrolysis DA peaks was not appreciably affected by varying the final concentration of perchloric acid between 0.2 and 1.0 *M* (data not shown). Decreased peak heights were seen outside this range. Consequently, a final concentration of 0.2 *M* perchloric acid was chosen for routine use in the post-column hydrolysis procedure.

Experiments in which the hydrolysis temperature was systematically varied indicated that the peak height of the free DA formed from DA-3-O-sulfate reached a plateau between 130° and 145°C, while DA-4-O-sulfate required a temperature of 145° for maximal hydrolysis (data not shown). The peak heights of both DA peaks formed from the DA-sulfate isomers increased approximately 20% with increased heating times between 0.7 and 1.8 min (data not shown).

Hydrodynamic voltammograms of free DA and DA-sulfate isomers were con-

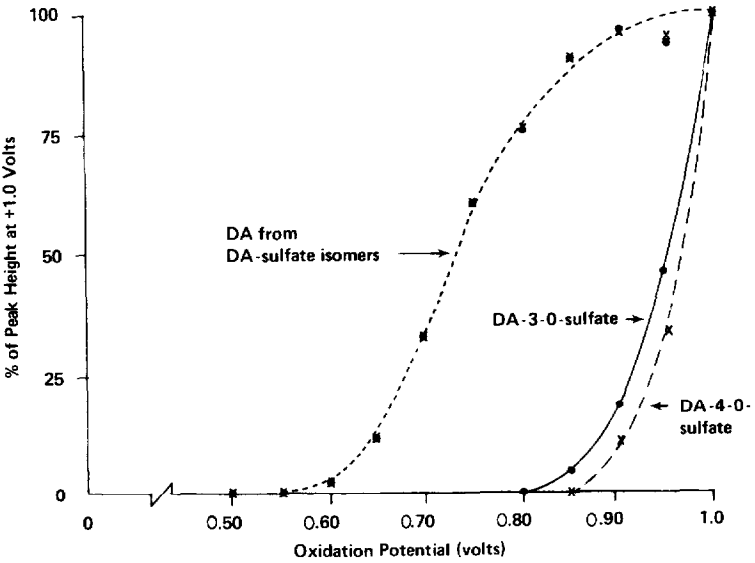

Fig. 4. Hydrodynamic voltammograms of free DA and DA-sulfate isomers. Conditions were as described in text. The free DA was derived from the post-column hydrolysis of either DA-3-O-sulfate (●) or DA-4-O-sulfate (×). The DA-sulfate peaks resulted from the injection of the appropriate isomer without post-column hydrolysis, *i.e.*, the hydrolysis coil was maintained at room temperature instead of at 145°C.

structed in order to select the optimal oxidation potential for detection of the hydrolysis products of DA-3-O-sulfate and DA-4-O-sulfate (Fig. 4). Conditions were as described in the methods section. The free DA on the voltammogram was derived from the post-column hydrolysis of DA-3-O-sulfate or DA-4-O-sulfate. The DA-3-O-sulfate and DA-4-O-sulfate peaks resulted from injection of the appropriate isomer while the hydrolysis coil was maintained at room temperature. An earlier experiment demonstrated that hydrolysis of either DA-sulfate isomer to free DA did not occur

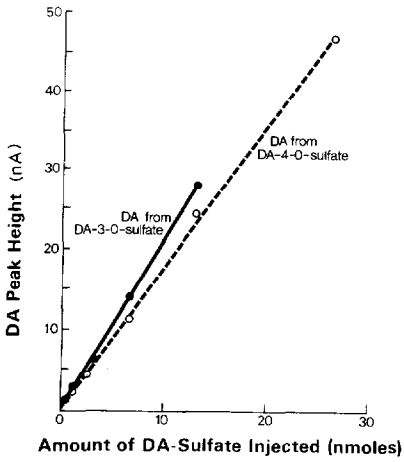

Fig. 5. Standard curve for post-column hydrolysis of DA-sulfate isomers. Conditions were as described in text.

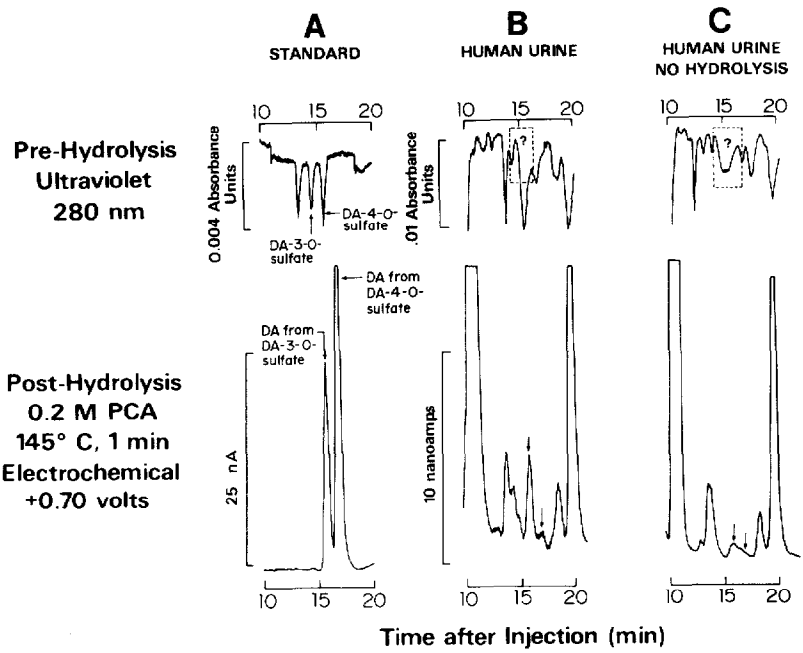

Fig. 6. Chromatograms of human urine injected directly onto the HPLC column. Conditions were as detailed in text. (A) Standard solution containing approximately 5 nmoles of each DA-sulfate isomer. (B) Human urine (20 μl) injected directly onto the HPLC column. (C) The same urine sample as in (B) was injected directly onto the HPLC column, but the hydrolysis coil was maintained at room temperature instead of at 145°C.

under these conditions (Fig. 3B). The optimal oxidation potential for the electrochemical detection of free DA derived from the post-column hydrolysis of DA-sulfate isomers is approximately 0.80 V (*vs.* an Ag/AgCl reference electrode) on the glassy carbon electrode. Detection at this potential produces a free DA peak height approximately 75% of the maximum obtained at an oxidation potential of 1.0 V. A higher oxidation potential may be utilized if lower absolute limits of detection are required, but decreased specificity would be expected at the higher potential. Final choice of oxidation potential depends on the requirements for specificity and lower limits of detection.

Direct detection of trace amounts of DA-3-O-sulfate or DA-4-O-sulfate is difficult utilizing electrochemical detection. Under the conditions utilized in the present experiments, the hydrodynamic voltammogram of either isomer had not yet reached a plateau at an oxidation potential of 1.2 V, the most positive potential tested (data not shown).

Linearity of the method

Injection of varying amounts of DA-3-O-sulfate and DA-4-O-sulfate indicated that there was a linear correlation between the electrochemical detector response to free DA and the amount of each DA-sulfate isomer injected (Fig. 5). Consequently, this method could be used to quantitate either of these DA-sulfate isomers in bio-

logical tissues or fluids. This laboratory has previously shown that the ultraviolet detector response to the unhydrolyzed DA-sulfate isomers is linear[6].

Example of use

Injection of human urine directly onto the HPLC column produced a peak after the post-column hydrolysis procedure which co-chromatographed exactly with the peak formed after injection of DA-3-O-sulfate onto the column. The peak corresponding to the hydrolysis product of DA-3-O-sulfate was not formed when water was substituted for acid in the hydrolysis procedure, or when the reaction was done at room temperature (Fig. 6).

CONCLUSIONS

This report has described a post-column hydrolysis technique for the determination of DA-3-O-sulfate and DA-4-O-sulfate, which should prove useful for investigations concerning the specific conjugates of DA in body tissues and fluids. It should also prove useful for studies utilizing labelled compounds to investigate possible metabolic roles of DA-3-O-sulfate and DA-4-O-sulfate.

The post-column hydrolysis technique has general applications for the analysis of other compounds that undergo acid or alkaline hydrolysis to form electrochemically active species. These include other conjugated catecholamines or indoles, their metabolites, and drug metabolites. The procedure is more rapid than the classical acid-hydrolysis procedures. Furthermore, it is possible to distinguish isomeric forms of the same conjugated compound using this technique, since the isomers are separated by HPLC before they are hydrolyzed.

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