*Journal of Chromatography*, 430 (1988) 263-269
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4272

# PREPARATION OF DOPAMINE 3-O-SULPHATE AND DOPAMINE 4-O-SULPHATE AS REFERENCE SUBSTANCES AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TRACE DETERMINATION

ELISABETH KIENZL

*Ludwig Boltzmann-Institute of Clinical Neurobiology, Wolkersbergenstrasse 1, A-1130 Vienna (Austria)*

and

KARL EICHINGER*

*Department of Chromatography and Spectroscopy, Institute of Organic Chemistry, Technical University of Vienna, Getreidemarkt 9, A-1060 Vienna (Austria)*

(First received March 11th, 1988; revised manuscript received April 21st, 1988)

## SUMMARY

Simple syntheses of the biologically important but hitherto difficult to obtain dopamine sulpho conjugates dopamine 3-O-sulphate (I) and dopamine 4-O-sulphate (II), as analytical reference substances, starting from dopamine hydrochloride are described. A method for the determination of I and II with reversed-phase high-performance liquid chromatographic separation and coulometric detection in human urine together with calibration and current–voltage curves are presented. Detection limits of approximately 100 pg of I or II and unequivocal substance identifications even in very complex substrates such as human urine are reported.

## INTRODUCTION

Changes in the activity of the dopaminergic system have been implicated in many physiological and clinical effects. Mainly neurophysiological states are related to the function of its metabolic pathways via monoamine oxidase (MAO), catechol O-methyltransferase or phenol sulphotransferase. Rapid sulpho conjugation of dopamine appears to occur predominantly in the blood stream and leads to dopamine 3-O-sulphate (I) and dopamine 4-O-sulphate (II) (Fig. 1). Therefore, the sulpho conjugates I and II might be better markers of dopamine release than the free dopamine [1].

0378-4347/88/$03.50 © 1988 Elsevier Science Publishers B.V.

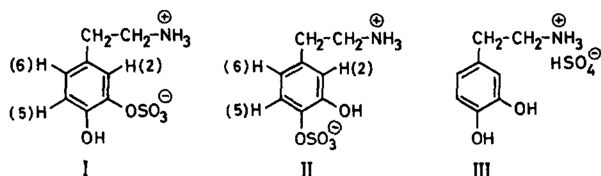


Fig. 1. Structures of dopamine 3-O-sulphate (I), dopamine 4-O-sulphate (II) and dopamine hydrogensulphate (III).

Many studies concerning the determination of I and II in blood, urine and cerebrospinal fluid [2–20], in adrenals of mammalian species and humans [21], in diverse biological materials [22], in cockroaches [23,24] and lobsters [25] and in connection with the activity of the enzyme phenol sulphotransferase [26,27] have been reported.

Despite this extensive interest in the determination of I and II, there is still no commonly accepted analytical method and the reported values for concentrations and the relative amounts of I and II in similar substrates differ widely [18,19,28–31].

Major disadvantages of the known analytical methods for the determination of I and II in biological substrates are the difficult availability of I and II as reference substances [19,23,32,33], high detection limits and insufficient chromatographic resolution in complex biological substrates such as urine [18,19], tedious and time-consuming procedures [23,32,34] and unconvincing structure identifications [30,33,35–37].

Hence a reliable study of the excretion pattern of I and II in human subjects depends on the development of an analytical method that combines sensitivity of detection with unequivocal substance identification, simplicity and speed.

## EXPERIMENTAL

### *Chemicals and reagents*

Chemicals for syntheses were of reagent grade and chemicals for analyses were of analytical-reagent grade. All chemicals were purchased from Merck (Darmstadt, F.R.G.) except dopamine hydrochloride, which was obtained from Sigma-Chemie (Deisenhofen, F.R.G.).

### *Equipment*

The reversed-phase high-performance liquid chromatographic (HPLC) determinations were carried out using a Model 5000 solvent delivery module (ESA, Bedford, MA, U.S.A.) and a Coulochem 5100 A detector (ESA) with a 5010 analytical cell (ESA) connected to a CI-10 recording integrator (LDC/Milton Roy, Riviera Beach, FL, U.S.A.). Potentials were +0.35 V (detector 1), +0.52 V (detector 2) and +0.35 V (guard cell), or a UV–VIS detector was used as described below.

Ion-exchange HPLC was performed with a Model 830 pneumatic solvent pump (DuPont, Wilmington, DE, U.S.A.) and a Spectromonitor 3100 UV–VIS detec-

tor (LDC). Columns (250 mm×4.6 mm I.D.) packed with S5 ODS (5 μm) (reversed phase) or S10 SAX (5 μm) (ion exchange) stationary phases (Spherisorb, Norwalk, CT, U.S.A.) were used. UV absorbance was measured at a wavelength of 280 nm. All samples were injected via a 100-μl loop and a rotary valve (Rheodyne, Cotati, CA, U.S.A.). $^1$H NMR spectra (250 and 90 MHz) were recorded with a Spectrospin 250-MHz spectrometer (Bruker, Karlsruhe, F.R.G.) and with an FX90Q instrument (JEOL, Tokyo, Japan), respectively.

*Chromatographic conditions*

For reversed-phase HPLC the mobile phase (pH 2.8) was a solution of 1.43 ml (15 mmol) of anhydrous acetic acid, 250 mg of sodium 1-heptanesulphonate and 80 mg of EDTA in 1000 ml of HPLC-grade water (LiChrosolv Merck). For ion-exchange HPLC the mobile phase contained 6.9 g of sodium dihydrogenphosphate, 250 mg of sodium 1-heptanesulphonate, 80 mg of EDTA and 30 ml of methanol in 970 ml of HPLC-grade water (LiChrosolv Merck), and was adjusted to pH 3.6 with phosphoric acid. The flow-rate of the mobile phase was 1.0 ml/min in each instance. Prior to use the mobile phases were filtered through a 0.22-μm membrane filter (Sartorius, Göttingen, F.R.G.). Peak identifications were performed by admixture with authentic standards.

*Syntheses and preparations of standards*

*Attempted preparation of dopamine 3-O-sulphate (I) and isolation of dopamine hydrogensulphate (III).* A 0.4-g (2.1-mmol) amount of anhydrous dopamine hydrochloride and 1.1 ml of concentrated sulphuric acid were reacted and worked up exactly according to the procedure of Jenner and Rose [32] for the preparation of I; 50 mg of a white hygroscopic powder were obtained. This substance gave a negative test for chloride. Its 250-MHz $^1$H NMR spectrum was not consistent with the structure of I but with III (Fig. 1). 250-MHz $^1$H NMR [$^2H_2O$–sodium 3-(trimethylsilyl)propanesulphonate]: $\delta$=2.87 [2H, t, $J$=7 Hz, Ar–$CH_2$], 3.23 [2H, t, $J$=7 Hz, $CH_2$–$NH_3^+$ ], 6.74 [1H, dd, $J$=8 and 2 Hz, H(6)], 6.86 [1H, d, $J$=2 Hz, H(2)], 6.92 [1H, d, $J$=8 Hz, H(5)] ppm.

*Dopamine 4-O-sulphate (II).* A 5.0-g (26.5-mmol) amount of dopamine hydrochloride was added with vigorous stirring during 2 h to 18 ml of concentrated sulphuric acid at −10°C. Stirring was continued at −10°C until the evolution of hydrogen chloride ceased. The reaction mixture was poured with stirring on 30 g of crushed ice and after a few minutes a white precipitate appeared. The mixture was stored at −4°C for 16 h and then suction filtered. The precipitate was washed with ice-cold water and recrystallized from water at 55°C. After drying over concentrated sulphuric acid in a vacuum desiccator, 0.5 g (8.1%) of II, m.p. 275–280°C, was obtained (Arora et al. [38] reported m.p. 273–274°C and Harbeson et al. [39] m.p. 257–259°C). $^1$H NMR ($d_6$-DMSO–trimethylsilyl): $\delta$=2.73 [2H, t, $J$=7 Hz, Ar–$CH_2$], 3.00 [2H, t, $J$=7 Hz, $CH_2$–N], 6.64 [1H, dd, $J$=8 and 2 Hz, H(6)], 6.74 [1H, d, $J$=2 Hz, H(2)], 7.09 [d, $J$=8 Hz, H(5)], ca. 8.0 [3H, very broad, s, –$NH_3^+$ ] ppm; Ar–OH not visible.

*Preparation of a mixture of dopamine 3-O-sulphate (I) and dopamine 4-O-sulphate (II) as a chromatographic standard.* To an ice-cold solution of 25 ml of anhydrous pyridine in 50 ml of chloroform were added 10 ml of chlorosulphonic acid with stirring at 0°C and then 10 g of dopamine hydrochloride in small portions. The reaction mixture was stirred at room temperature for 60 h and then poured on ice. After separation of the chloroform layer the aqueous phase was adjusted to pH 4.0 with sodium hydroxide and evaporated to dryness under reduced pressure. The residue gave a 90 MHz $^1H$ NMR spectrum [$^2H_2O$–sodium 3-(trimethylsilyl)propanesulphonate] consistent with an approximately equimolar mixture of I and II in comparison with the corresponding spectra described by Osikowska et al. [34]. Reversed-phase HPLC showed only two peaks with UV or coulometric detection.

### *Sample preparation for HPLC*

Standards and urine samples were pressure-filtered through ACRO LC25 0.45-µm filter (Gelman, Ann Arbor, MI, U.S.A.) prior to injection. No further sample preparation was necessary.

## RESULTS AND DISCUSSION

### *Syntheses of standards and peak identifications in HPLC*

In particular to overcome the analytical problems mentioned above, we first investigated the availability of I and II as reference substances.

Most workers [18,19,23,30,35] used the standard preparation of I and II reported by Jenner and Rose [32]. The crucial step in this procedure is a preparative ion-exchange chromatographic separation of the presumed I and II after reaction of dopamine hydrochloride with concentrated sulphuric acid and work-up.

Elaborate efforts by Osikowska et al. [34] were not successful in repeating the synthesis according to Jenner and Rose [32], indicating doubt about the claims of all those workers reporting of having isolated I and II. Likewise, both we and Wesemann [40] could not repeat the procedure of Jenner and Rose [32]. The results of our experiments rather suggested the isolation of the dopamine salt III instead of I.

To the best of our knowledge, only Hegedüs [41] has reported an unequivocal synthesis of I, but this was considered impractical because of its many synthetic steps and extreme low overall yield. As the only easily obtainable precursor of I is dopamine hydrochloride and the regiospecific sulphation of its 3-OH group has been found to be an unsolved synthetic problem, we attempted a short synthesis of an authentic mixture of I and II starting from dopamine hydrochloride. A mixture of chlorosulphonic acid and pyridine in chloroform was found to be an excellent reagent for the production of a nearly equimolar mixture of I and II. The structures and quantitative relationships of these two components were determined by comparison of its $^1H$ NMR spectrum with that of II and the corresponding data of Osikowska et al. [34] for I.

Harbeson et al. [39] developed a three-step synthesis of II starting from do-

pamine. Simpler still is the one-step synthesis of II of Arora et al. [38] from dopamine hydrochloride and concentrated sulphuric acid. They reported that very pure dopamine had to be used as starting material otherwise crystalline II often could not be obtained. We repeated this synthesis many times, following closely the described procedure. Even with the purest commercially available dopamine hydrochloride as starting material, crystalline II was never obtained.

Because of its potential simplicity, we investigated the synthesis of Arora et al. [38] in detail and found a successful modification. A reaction temperature of $-10°C$ instead of $0°C$, a reaction time of 2 h instead of 10 min and pouring the reaction mixture on ice stead of adding ice to it always gave pure and crystalline II even with the usual commercial dopamine hydrochloride as starting material.

*Chromatography*

According to our chromatographic results the reaction mixture obtained when following the procedure of Jenner and Rose [32] for the preparation of I and II consists predominantly of III with smaller amounts of I and II. A typical chromatogram is presented in Fig. 2. Even with ion-exchange HPLC and UV detection as detailed above, I and II were not separable. Therefore, it seems likely that most of the discrepancies in the literature concerning the preparation of I and II according to Jenner and Rose [32] have their origin in a confusion of III with I and II.

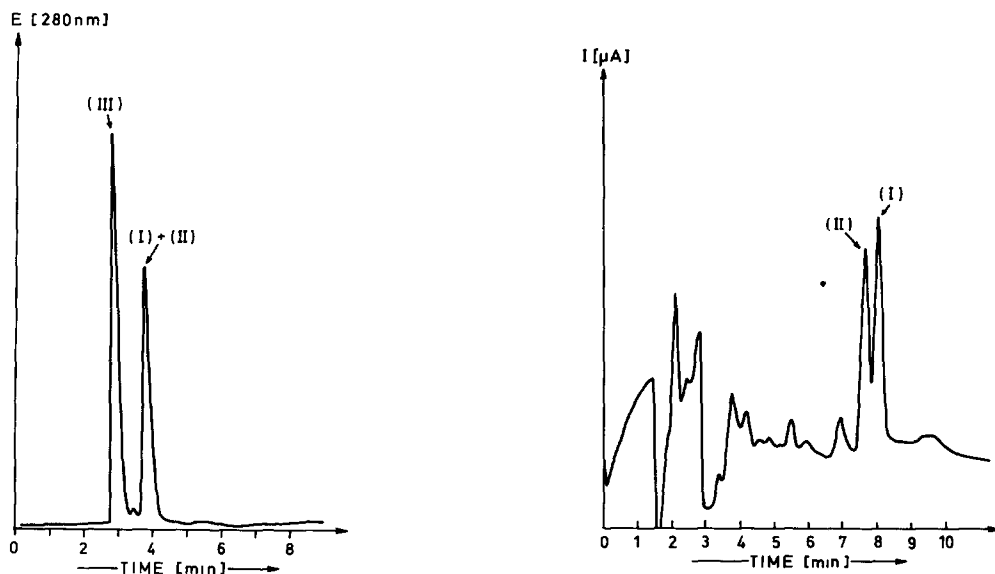


Fig. 2. Ion-exchange chromatograms with UV detection (conditions as in text) of a reaction mixture obtained according to Jenner and Rose [32]. A 100-$\mu l$ volume containing approximately 3.0 $\mu g$ of reaction mixture were injected. $k'$ values: III, 2.52; I and II, 3.98.

Fig. 3. Reversed-phase chromatogram with coulometric detection of human urine from a normal adult, diluted 1:100. A 100-$\mu l$ volume was injected. $k'$ values: I, 4.00; II, 3.75. Amounts found: 1.45 ng of I and 1.34 ng of II.

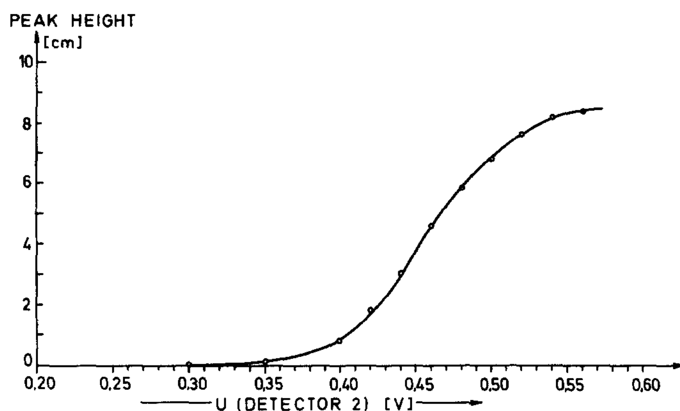


**Fig. 4. Peak height versus detector (2) voltage for 5 ng of II. I gives an almost identical curve.**

A successful separation of I and II (from urine samples) was achieved by reversed-phase HPLC as described above. A representative chromatogram is shown in Fig. 3. Under these selected conditions, III has a far higher $k'$ value and does not interfere with the determination of I and II. At pH 3–10, I and II are stable for at least several days at room temperature in pure aqueous solutions and as standards in human urine.

The calibration graphs for the coulometric detection of I and II in the range of interest for the analysis of human urine were almost identical and followed the equation $y=A+Bx$, where $y$=peak height (mm), $x$=amount of I or II (ng), $A=4.62$, $B=16.81$ and the correlation coefficient $r=0.995$ from 0.5 to 5.0 ng of I or II. According to our results, normal adults (three males, one female) excrete approximately 1–3 mg of I per litre of urine in 24 h and nearly that amount of II.

As the sensitivity of the coulometric detector is a function of the detection voltage, current–voltage curves for I and II were generated. A typical curve is presented in Fig. 4 for 5 ng of II. A potential of 0.50–0.55 V was found to give an optimum signal-to-noise ratio; higher voltages led to an intolerably high current and an unstable baseline. I and II showed virtually identical behaviour.

According to additional results with a higher detector gain, a detection limit of approximately 100 pg of I or II is easily achieved, even with complex biological substrates such as human urine. A detection limit as low as 20 pg should be possible in sufficiently simple substrates if the detector gain is set to the maximum. Application of this approach will be essential to continuing investigations of sulphate conjugation in the metabolism of dopamine in the central and peripheral nervous system.

ACKNOWLEDGEMENTS

The authors are much obliged to Professor Dr. Kurt Jellinger (Ludwig Boltzmann-Institute of Clinical Neurobiology, Vienna, Austria) for financial support and valuable suggestions, to Professor Dr. Peter Riederer (Clinical Neurochemistry, Psychiatric Hospital, University of Würzburg, Würzburg, F.R.G.) for helpful suggestions and to Dr. Gerhard Schulz and Dr. Peter Nussbaumer (Sandoz Research Institute, Vienna, Austria) for recording the 250-MHz $^{1}H$ NMR spectra.

## REFERENCES

1 O. Kuchel, N.T. Buu, K. Racz, A. De Lean, O. Serri and J. Kyncl, Fed. Proc., Fed. Am. Soc. Exp. Biol., 45 (1986) 2254.
2 M. Oka, Y. Ishimura, T. Tsunematsu, K. Minakuchi, T. Ohochi and K. Matsumoto, Biochem. Pharmacol., 36 (1987) 3205.
3 A. Yamatodani, T. Yamamoto, K. Hashizume and H. Wada, Med. Philos., 6 (1987) 475.
4 R.-K. McCulloch, R. Vandongen, A.M. Tunney, L.J. Beilin and P.B. Rogers, Am. J. Physiol., 253 (1987) E312.
5 G.M. Tyce, R.A. Van Dyke, S.R. Rettke, S.R. Atchison, R.H. Wiesner, E.R. Dickson and R.A. Krom, J. Lab. Clin. Med., 109 (1987) 532.
6 J. Odink, E.J. Van der Beek, H. Van den Berg, J.J.P. Bogaards and J.T.N.M. Thissen, Int. J. Sports Med., 7 (1986) 352.
7 L.A. Toth and M.A. Elchisak, J. Pharmacol. Exp. Ther., 240 (1987) 359.
8 M.-C. Scott and M.A. Elchisak, J. Chromatogr., 413 (1987) 17.
9 T. Yamamoto, Osaka Daigaku Igaku Zasshi, 38 (1986) 37.
10 P.G. Swann and M.A. Elchisak, J. Chromatogr., 381 (1986) 241.
11 C.M. Scott, Diss. Abstr. Int. B, 47 (1986) 150.
12 L.A. Toth, M.C. Scott and M.A. Elchisak, Life Sci., 39 (1986) 519.
13 G.N. Tyce, J.M. Messik, T.L. Yaksh, D.E. Byer, D.R. Danielson and D.K. Rorie, Fed. Proc., Fed. Am. Soc. Exp. Biol., 45 (1986) 2247.
14 T. Tsunematsu and T. Tokushima, J. Exp. Med., 32 (1985) 23.
15 O. Kuchel, C. Hausser, N.T. Buu and S. Tenneson, J. Neural Transm., 62 (1985) 91.
16 T. Yamamoto, A. Yamatodani, M. Nishimura and H. Wada, J. Chromatogr., 342 (1985) 261.
17 Y. Ishimura, T. Ohuchi, T. Tsunematsu, K. Mizuguchi and M. Oka, Rinsho Yakuri, 15 (1984) 13.
18 Y. Arakawa, K. Imai and Z. Tamura, J. Chromatogr., 162 (1979) 311.
19 R.L. Bronaugh, S.E. Hattox, M.M. Hoehn, R.C. Murphy and C.O. Rutledge, J. Pharmacol. Exp. Ther., 195 (1975) 441.
20 I. Merits, Biochem. Pharmacol., 25 (1976) 829.
21 K. Racz, O. Kuchel and Nguyen Thanh Buu, J. Comp. Physiol. B, 155 (1985) 367.
22 M.A. Elchisak, Prog. HPLC, 2 (1987) 329.
23 R.P. Bodnaryk and P.C.J. Brunet, Biochem. J., 138 (1974) 463.
24 B.D. Sloley and R.G.H. Downer, Insect Biochem., 17 (1987) 591.
25 M.B. Kennedy, J. Neurochem., 30 (1978) 315.
26 S. Demassiex, L. Bordeleau, D. Gravel and S. Carriere, Life Sci., 40 (1987) 183.
27 L.A. Toth and M.A. Elchisak, Life Sci., 40 (1987) 473.
28 W.N. Jenner and F.A. Rose, Nature (London), 257 (1974) 237.
29 M. Sandler, R.D. Johnson, C.R.J. Ruthven and B.L. Goodwin, in W. Birkmayer and O. Hornykiewicz (Editors), Advances in Parkinsonism, Editiones Roche, Basle, 1976, p. 344.
30 M.A. Elchisak and J.H. Carlson, Life Sci., 30 (1982) 2325.
31 K.P. Wong, C.R.J. Ruthven and M. Sandler, Clin. Chim. Acta, 47 (1973) 215.
32 W.N. Jenner and F.A. Rose, Biochem. J., 135 (1973) 109.
33 M.A. Elchisak, J. Chromatogr., 255 (1983) 475.
34 B.A. Osikowska, J.R. Idle, F.J. Swinbourne and P.S. Sever, Biochem. Pharmacol., 31 (1982) 2279.
35 D.S. Eggleston, D.F. Chodosh, T. Jain, C. Kaiser and D.M. Ackermann, Acta Crystallogr., Sect. C, 41 (1985) 76.
36 M.A. Elchisak, J. Chromatogr., 264 (1983) 119.
37 M.A. Elchisak, Fed. Proc. Fed. Am. Soc. Exp. Biol., 45 (1986) 2241.
38 S.K. Arora, B.S. Cho and D.L. Venton, Synthesis, (1984) 884.
39 S.L. Harbeson, D.J. Kerkman and J.F. DeBernardis, Org. Prep. Proced. Int., 15 (1983) 243.
40 W. Wesemann, University Marburg/Lahn, F.R.G., personal communication, 1987.