Journal of Chromatography, 383 (1986) 132—136
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3310

Note

Preparative separation of polar unstable compounds (catecholamines) from a synthetic mixture by high-speed counter-current chromatography

ADRIAN WEISZ and SANFORD P. MARKEY*

Laboratory of Clinical Science, Section on Analytical Biochemistry, National Institute of Mental Health, Bethesda, MD 20892 (U.S.A.)

and

YOICHIRO ITO

Laboratory of Technical Development, National Heart, Lung, and Blood Institute, Bethesda, MD 20892 (U.S.A.)

(First received May 16th, 1986; revised manuscript June 30th, 1986)

Mass spectrometric (MS) studies on the metabolism and kinetics of the neurotransmitter norepinephrine (NE) require the use of a stable isotopomer of this compound. The preparation of various deuterated (ring and side-chain) and ^{13}C-labelled (side-chain) species has been reported earlier [1—3]. We desired a multiple ring-labelled NE for studies in humans, one which would retain its isotopic content during metabolism to oxidized metabolites as well as during chemical manipulations. Furthermore, MS fragmentation of a ring-labelled species would contain mass-shifted ions at high *m/z* useful for quantitative analysis making this a useful internal standard for sensitive MS assays. [Phenyl-$^{13}C_6$]guaiacol was used as the starting material as outlined in the scheme previously reported [4, 5]. Inadvertently in the last step of the synthesis with one batch of starting material, the hydrogenation was too vigorous and a mixture of NE and dopamine (DA) (3:1) was obtained (Fig. 1).

Most of the procedures described in the literature for quantitative separation of NE from DA deal with isolation of these catecholamines from biological materials and therefore relate to quantities in the pico- to microgram range

[6—8]. Since we had more than 100 mg of the NE—DA mixture to separate, none of the techniques previously described for this purpose applied.

Reviewing the growing number of applications of counter-current chromatography (CCC) for the isolation and purification of different classes of compounds, we considered it to be a good choice for our synthetic mixture [9, 10]. CCC is particularly useful in the preparative range (milligram to gram) and it is especially advantageous for polar substances [9, 11]. Because of the absence of solid supports, the recovery is maximal. The time required for quantitative separation in high-speed CCC is no more than a few hours instead of a few days usually required in droplet CCC [12]. These are especially important considerations for perishable compounds such as catecholamines.

This paper describes the application of high-speed CCC in the separation of NE and DA produced by catalytic reduction of noradrenalone.

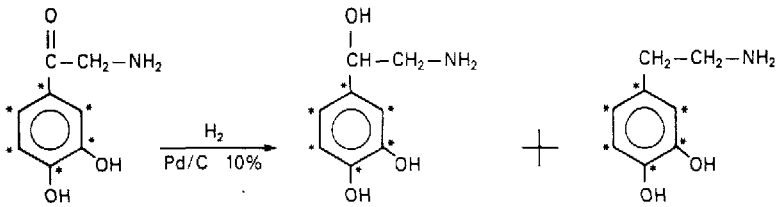

Fig. 1. Catalytic reduction of noradrenalone to obtain a mixture (3:1) of norepinephrine (NE) and dopamine (DA).

EXPERIMENTAL

Materials

The hydrochloride salts of D,L-NE and DA for the test sample mixture separation were purchased from Sigma (St. Louis, MO, U.S.A.). The [phenyl-$^{13}C_6$]NE and -DA hydrochloride salts mixture was obtained from the above mentioned catalytic reduction of [phenyl-$^{13}C_6$] noradrenalone. Barium chloride dihydrate 99+% ($BaCl_2 \cdot 2H_2O$) was purchased from Aldrich (Milwaukee, WI, U.S.A.). *n*-Butanol used for CCC was of a chromatographic grade. The other chemicals were analytical grade.

Counter-current chromatography

High-speed CCC is a new form of liquid—liquid partition method which utilizes an intriguing hydrodynamic behavior of two immiscible solvent phases through a coiled tube subjected to a particular type of planetary motion [13]. Both retention of the stationary phase and phase mixing are achieved under continuous elution in a long (over 100 m) tubular space free of solid support, thus resulting in a chromatographic separation of various biological samples [14] without complications arising from the use of solid support.

In this experiment high-speed CCC separations were performed with a multilayer coil planet centrifuge described in detail earlier [13]. Similar equipment is commercially available from either P.C. Inc., Potomac, MD, U.S.A. or Pharma-Tech Research Corporation, Baltimore, MD, U.S.A.

A two-phase solvent system was prepared by equilibrating equal volumes of *n*-butanol and saturated $BaCl_2$ aqueous solution (pH 6.5) in a separatory funnel at room temperature. Initially, a saturated sodium chloride aqueous solution was used as the mobile phase with which excellent separations of NE and DA were obtained. However, due to difficulties in recovering NE from the sodium chloride solution, $BaCl_2$ solutions were used. High ionic concentrations are required to enhance the organic/aqueous partition of the catecholamines. The partition coefficient (C_{aq}/C_{nonaq}) of the sample in the above solvent system measured 4.95 for NE and 2.26 for DA. Both test sample mixture (147 mg NE + 43 mg DA) and crude sample (ca. 100 mg) were each dissolved in about 6 ml of the solvent system consisting of equal volumes of the upper and the lower phases. The saturated $BaCl_2$ solution was filtered to remove salt particles prior to use.

In each separation the column was first entirely filled with the stationary upper phase followed by injection of the sample solution through the sample port. Then, the mobile phase was pumped into the column through the internal head end at a flow-rate of 125 ml/h using a Beckman Accu-Flo pump, while the column was spun at 635 rpm. The eluate from the outlet of the column was continuously monitored with an LKB Uvicord S at 278 nm and then fractionated into test tubes with an LKB fraction collector at 1-min intervals for further analysis.

Recovery of the sample

After reuniting the fractions belonging to each peak, the solvent (water) was evaporated by freeze-drying. The white residue thus obtained was washed (3 × 20 ml) with warm (~ 50°C) absolute ethanol. The ethanol was removed with a rotary evaporator. The residue was redissolved in absolute ethanol (~ 10 ml) at room temperature. After evaporating the ethanol, the product was obtained free of $BaCl_2$. The recovery of NE from $BaCl_2$ solutions was 86%.

Mass spectrometry

Electron-impact mass spectra of the recovered derivatized [trimethylsilylated with bis(trimethylsilyl)trifluoroacetamide (BSTFA)] NE and DA were recorded with a Finnigan 3200 gas chromatograph—mass spectrometer operated under computer control (Teknivent). Typical analysis conditions were: gas chromatography (GC) column, 1% OV-1, 80—100 mesh; column temperature, 120—250°C at 10°C/min; helium flow-rate, 15 ml/min; electron-impact ionization, 70 eV ionizing energy. Fragmentation patterns of $^{13}C_6$-labelled catecholamines: tetra(trimethylsilyl)-NE: 463 (0.2, M^+), 448 [3.0, $(M-CH_3)^+$], 361 [100.0, $(M-CH_2NHTMS)^+$], 273 [7.3, $(M-CH_2NHTMS-Si(CH_3)_4)^+$], 102 (20.4, CH_2^+NHTMS); tetra(trimethylsilyl)-DA: 447 (0.3, M^+), 432 [8.7, $(M-CH_3)^+$], 344 (3.7), 273 [2.2, $(M-CH_2N(TMS)_2)^+$], 185 [7.8, $(M-CH_2N(TMS)_2-Si(CH_3)_4)^+$], 174 (100.0, $CH_2^+N(TMS)_2$).

RESULTS AND DISCUSSION

When the test sample mixture of NE and DA was chromatographed with the coil planet centrifuge, two major peaks, fractions 62—81 and 90—120, were

well resolved and eluted within 2 h (Fig. 2). On separating the [$^{13}C_6$]noradrenalone reduction product mixture with the coil planet centrifuge, three major peaks, fractions 50—60, 75—92, and 130—155, were resolved and eluted within 3 h (Fig. 3). The identification of the catecholamines was made by high-performance liquid chromatography with electrochemical detection (HPLC—ED) which confirmed the purity of CCC-isolated products [15]. The peak at 2.25 h (Fig. 3) remains unidentified. GC—MS analysis of trimethylsilyl derivatives also indicated the CCC-separated products were free from contamination and were completely resolved. The mass spectra were characteristic of derivatized DA and NE, but shifted by 6 daltons due to ^{13}C substitution in fragments containing the catechol portion of the molecule (e.g., M^+ or $(M—CH_2NHTMS)^+$), and unshifted for other ions derived from the side-chain portion of the molecule (e.g., CH_2^+NHTMS, or $CH_2^+N(TMS)_2$).

A most significant advantage of CCC is the nearly quantitative recovery of separated products. In test runs, 97% of NE and 90% of DA applied to CCC were recovered in combined fractions, as determined by HPLC—ED. Although CCC has been used for purification of synthetic peptides [16], the above experiment is the first described application for quantitative separation and purification of a synthetic organic mixture. In conclusion, rapid and complete separation of the two catecholamines from a crude mixture shows that the

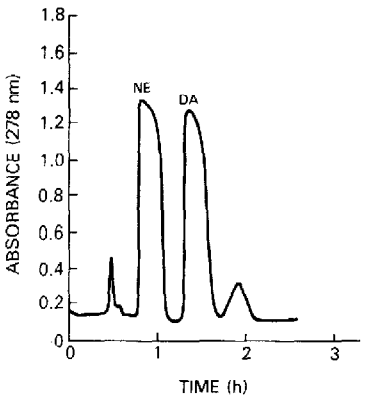

Fig. 2. Separation of a test mixture of norepinephrine (NE) and dopamine (DA) by high-speed counter-current chromatography.

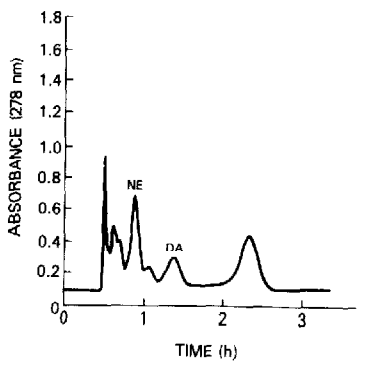

Fig. 3. Separation of [$^{13}C_6$]noradrenalone reduction products by high-speed counter-current chromatography. Peaks: NE = [$^{13}C_6$]norepinephrine; DA = [$^{13}C_6$]dopamine.

high-speed CCC technique has great potential in the organic chemists' arsenal for the separation of mixtures.

ACKNOWLEDGEMENTS

The authors are grateful to Mr. G. Weisz for fruitful suggestions and to Mr. J. Savitt and Mr. J. Bacon for performing the HPLC measurements.

REFERENCES

1 R.C. Murphy, J. Labelled Compd., 11 (1975) 341.
2 A. Kalir, C. Freed, K.L. Melmon and N. Castagnoli, Jr., J. Labelled Compd. Radiopharm., 13 (1977) 41.
3 T. Suzuki, S. Sakada, M. Ueji and S. Kishimoto, Life Sci., 36 (1985) 435.
4 J.R. Heys, D.H. Chew and J.H. Saugier, J. Labelled Compd., Radiopharm., 19 (1982) 801.
5 A. Weisz and S.P. Markey, 2nd International Symposium on the Synthesis and Applications of Isotopically Labeled Compounds, Kansas City, KY, Sept. 3—6, 1985, Abstract PB-34.
6 S. Higa, T. Suzuki, A. Hayashi, I. Tsuge and Y. Yamamura, Anal. Biochem., 77 (1977) 18.
7 C. Atack and T. Magnusson, Acta Pharmacol. Toxicol., 42 (1978) 35.
8 B.P. Sleckman and J. Sherma, J. High Resolut. Chromatogr., Chromatogr. Commun., 6 (1983) 156.
9 W.D. Conway and Y. Ito, LC, Liq. Chromatogr. HPLC Mag., 2 (1984) 368.
10 Y. Ito, Adv. Chromatogr., 24 (1984) 181.
11 J.L. Sandlin and Y. Ito, J. Chromatogr., 348 (1985) 131.
12 T. Tanimura, J.J. Pisano, Y. Ito and R.L. Bowman, Science, 169 (1970) 54.
13 Y. Ito, J. Sandlin and W.G. Bowers, J. Chromatogr., 244 (1982) 247.
14 Y. Ito, Critical Review Journal in Anal. Chem., CRC Press, Inc., 1986, in press.
15 C.C. Chiueh, Z. Zukowska-Grojec, K.L. Kirk and I.J. Kopin, J. Pharmacol. Exp. Ther., 225 (1983) 529.
16 M. Knight, Y. Ito, P. Peters and C. diBello, J. Liq. Chromatogr., 8 (1985) 2281.