*Journal of Chromatography*, 533 (1990) 166–170
*Biomedical Applications*
Elsevier Science Publishers B V , Amsterdam

CHROMBIO 5487

# Note

## Determination of plasma catecholamines via condensation with diphenylethylenediamine: simplification of the procedure

PETR HUŠEK*, JITKA MALÍKOVÁ and GERTRUDA HERZOGOVÁ

*Research Institute of Endocrinology, Národní třída 8, CS 11694 Prague 1 (Czechoslovakia)*

(First received May 22nd, 1990, revised manuscript received July 23rd, 1990)

Catecholamines (CAs) exhibit a native fluorescence when irradiated with UV light of 285 nm. However, the intensity of the emitted light is weak and the sensitivity limit is about 300 pg [1,2], which is insufficient for the measurement of the plasma levels of CAs [3]. As an alternative, a post-column derivatization to trihydroxyindoles (THI) was elaborated [4,5], but the sensitivity for dopamine (DA) was low. During the late 1980s, attempts were made to refine the most often used technique for CA assay, liquid chromatography with electrochemical detection (LC–ED), in order to obtain the sensitivity needed for the measurement of the extremely low levels of unconjugated plasma DA [6–10]. Doubts remain if the commonly used glassy carbon electrodes are the best detection tool as the less convenient carbon paste electrode was found to be superior in this respect [11].

A promising solution was found in the use of highly fluorescent derivatives obtained by condensation of CAs with 1,2-diphenylethylenediamine (DPE). This approach was first recommended by Mitsui *et al.* [12] and studied comprehensively more recently [10]. We have been concerned with the same problem since 1986 and relevant results are reported in this paper.

## EXPERIMENTAL

### *Chemicals*

Norepinephrine (NE), epinephrine (E), isoproterenol (IP) and DA were obtained from Sigma (St. Louis, MO, U.S.A ). Tris, ethylenediaminetetraacetic acid (EDTA), diphenylborate–ethanolamine complex (DFB), tetraoctylammonium bromide (TOAB) and potassium hexacyanoferrate(III) (PFC) were purchased from Fluka (Buchs, Switzerland). Alumina (Aluminiumoxid 90, acidic, 70–230 mesh) was obtained from Merck (Darmstadt, F.R.G.) and was activated by heating with 2 *M* perchloric acid at 95°C for 45 min and after washing with water was dried at 120°C for 1 h and at 200°C for 2 h. DPE was synthesized as recommended

0378-4347/90/$03 50 

by Irving and Parkins [13]. All other chemicals were of analytical-reagent grade from Lachema (Brno, Czechoslovakia).

*Procedures*

The solution for collection of human blood was prepared by dissolving 900 mg of EGTA in 5 ml of 1 *M* NaOH and, after addition of 600 mg of reduced glutathione and adjustment of the pH to 7–7.5, the volume was brought to 10 ml by addition of water. The solution was stored at −20°C and 0.1 ml was taken for collection of 5 ml of human blood in chilled tubes. CAs in plasma were isolated by a combined procedure of liquid–liquid extraction without the use of *n*-octanol and column alumina extraction [14].

To 0.5–1 ml of plasma, spiked with 2 pmol of IP in 5 μl of water, 0.5 ml of 2*M* ammonia–ammonium chloride buffer (pH 8.6) containing 0.2% DFB and 0.5% EDTA and 2.5 ml of extraction solvent, consisting of heptane–chloroform (4:1, v/v) containing 0.5% TOAB, were added. After shaking for 2 min and centrifugation (5 min, 1000 *g*), 2 ml of the organic phase were transferred into another glass tube and shaken with 0.5 ml of 0.2 *M* acetic acid for 2 min. About 400 μl of the lower aqueous phase were withdrawn, mixed with 500 μl of 1 *M* Tris buffer and applied to an alumina column for additional clean-up. For this purpose a micropipette tip, 300 μl in volume, was filled with about 20 mg of alumina, fixed by means of soft synthetic wool, and the solution was drawn through by suction using a peristaltic pump at flow-rate of *ca.* 1 ml/min. After washing with 2 ml of distilled water, CAs were eluted by forcing 150 μl of 70 m*M* citric acid–acetonitrile (1:1, v/v) through the column by means of a syringe.

For condensation of CAs with DPE, the whole volume of the eluate was mixed with 15 μl of 100 m*M* methanolic DPE solution and 15 μl of 10 m*M* PFC in 1 *M* potassium hydrogenphosphate solution and the mixture was incubated at 40°C for 40 min. For condensation of a standard CA sample the acetonitrile was mixed 1:1 with 50 m*M* citric acid and incubated as described. The total reaction volume was injected into the chromatographic system after cooling in ice-cold water for about 5 min.

*Chromatography*

A Shimadzu (Kyoto, Japan) LC-6A pump with an on-line injector (200-μl loop) was connected with a 150 mm × 3.0 mm I.D. SGX $C_{18}$ column with 5-μm particle size ODS-silica (Tessek, Prague, Czechoslovakia) and a Shimadzu RF-530 fluorescence monitor with a xenon lamp. The mobile phase was acetone [or acetonitrile–acetone (1:1)]–50 m*M* citric acid–100 m*M* potassium hydrogenphosphate (2:1:1, v/v) at a flow-rate of 0.5 ml/min. The excitation and emission wavelengths were 350 and 480 nm, respectively.

RESULTS AND DISCUSSION

For the clean-up of plasma CAs, Mitsui *et al.* [12] used a strong cation exchanger, Toyopak SP (Toyo Soda, Tokyo, Japan), with a particle size of 19–40 μm. Working with a similar material of the Dowex or Amberlite type we could not achieve satisfactory results because the plasma column percolation was far from smooth. In similar work, Van der Hoorn *et al.* [10] also abandoned this approach to CA clean-up. Like them, we turned our attention to the liquid–liquid extraction method of Smedes *et al.* [15] to evaluate its usefulness in connection with the subsequent CA derivatization and fluorescence detection.

In the light of our findings and experience, the recently published approach [10] seems to be unnecessarily laborious, mainly because of the need (1) to prepurify the extraction assay buffer and *n*-octanol, (2) to repeat the extraction and to shake the organic solvent with the assay buffer additionally, (3) to use bicine buffer and additional chemicals for the reaction and (4) to reduce the volume injected and thus the of amount CAs subjected to detection to about one sixth of the total.

In our approach, a substantial improvement was achieved by using a rapid alumina column clean-up following the liquid–liquid extraction. Using this step, the interfering substance from plasma [10], which hinders the efficient derivatiza-

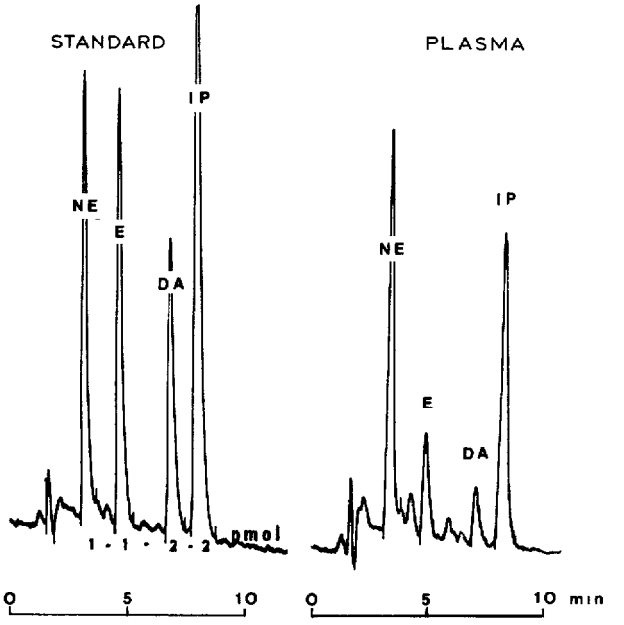


Fig 1. CA standards (1, 1, 2 and 2 pmol of NE, E, DA and IP, respectively) and unconjugated CAs from 1 ml of plasma analysed under the conditions given under Experimental after precolumn derivatization with DPE.

tion of the primary amines NE and DA, was removed. Moreover, using this subsequent step the whole volume of the eluate can be subjected to the derivatization and analysis. The overall recovery is a result of a two-fold volume reduction in the course of the liquid extraction ($0.8 \times 0.8 = 0.64v$) and yield of alumina sorption (*ca.* 75%); about 45–48% of the original amount of CAs are recovered.

Dissolution of DPE in methanol instead of in dilute hydrochloric acid [10,12] is advantageous with regard to the necessity to increase the pH to about 6. Such a neutralization can be achieved by dissolving PFC in potassium hydrogenphosphate instead of in water and, therefore, an additional increase in the total volume by adding a neutralizing agent is unnecessary. Van der Hoorn *et al.* [10] recommended further the use of bicine buffer for neutralization and optimum reaction yields, whereas with phosphate the assay was poor under their conditions. It is worth noting that, under our conditions, the yield was the highest with phosphate buffer and the use of bicine proved to give inferior results.

The whole reaction volume, being less than 200 $\mu$l, could be injected into the chromatographic system. The use of a mobile phase with a composition similar to that of the reaction medium proved to be advantageous. The replacement of toxic acetonitrile and methanol with acetone is also to be recommended and from Fig. 1 it can be seen that the separation efficiency and sensitivity of the assay are not affected by this modification.

The highest derivatization yields were obtained at pH 5.8–6.2. Neutralization

TABLE I

REPRODUCIBILITY OF THE PROCEDURE VERIFIED BY INTRA- AND INTER-ASSAY OF THE SAME POOLED PLASMA SAMPLE (A) AND SUBSEQUENTLY SPIKED WITH CA STANDARDS (B)

| Compound | Method (A) ($n$ = 7) | | | | Method B | |
|---|---|---|---|---|---|---|
| | Intra-assay | | Inter-assay | | Added (pg) | Found (pg) |
| | Mean (pg/ml) | R.S.D (%) | Mean (pg/ml) | R.S.D (%) | | |
| NE | 1.92 | 6.3 | 1.97 | 7.1 | 1.0 | 2.87 |
| | | | | | 5.0 | 7.01 |
| | | | | | 20 | 21.38 |
| E | 0.34 | 8.8 | 0.32 | 6.2 | 0.2 | 0.53 |
| | | | | | 1.0 | 1.31 |
| | | | | | 5 | 5.37 |
| DA | 0.21 | 23.8 | 0.23 | 17.4 | 0.2 | 0.40 |
| | | | | | 1.0 | 1.19 |
| | | | | | 5 | 5.26 |

was easily achieved by addition of phosphate containing the oxidizing agent PFC to the acidic eluate. The amount of phosphate added was sufficient to neutralize 50 m*M* citric acid, which was used for the condensation of CA standards directly in a tube. For elution of CAs from alumina columns it was necessary to increase the citric acid concentration to 70 m*M*, because residual Tris cannot be washed out from alumina by water and it partially neutralizes the citric acid in the eluent. The elution power of the eluent was not affected by the presence of acetonitrile and the elution volume of 150 μl was sufficient. The same is true for the amount of DPE in the reaction medium (1.5 μmol); a further increase did not improve the reaction yields. After condensation, the samples were stored in ice-cold water and refrigerated in the dark. Even when analysed within 2 h after the incubation no changes in the yield were observed over 8 h. The intra- and inter-assay variabilities, including the assay linearity, are given in Table I.

To conclude, our procedure can be considered as a modification of the original method of Mitsui *et al.* [12] and a simplification of the more recent approach [10] with some important improvements and advantages. It has been successfully used in our laboratory for the routine measurement of plasma CAs in hundreds of samples.

REFERENCES

1 Y Yui and C. Kawai, *J Chromatogr*, 206 (1981) 586
2 N G Abeling, A H. van Gennip, H Overmars and P A Voute, *Clin. Chim Acta*, 137 (1984) 211
3 B A. Davis, *J Chromatogr*, 466 (1989) 89
4 B Neidhart, J Ruter, Ch Lippmann, P Deutschmann and I Walker, *Fresenius' Z. Anal Chem.*, 323 (1986) 880
5 K Mori, *J Chromatogr*, 218 (1981) 631
6 A Guillemin, S. Troupel and A Galli, *Clin Chem*, 34 (1988) 1913
7 N. R Musso, C Vergassola, A Pende and G. Lotti, *Clin Chem*, 35 (1989) 1975
8 I. Meineke, E Stüwe, E M Henne, G Rusteberg, E Brendel and C DeMey, *J Chromatogr*, 493 (1989) 287.
9 P S. Rao, N. Rujikarn, J. M Luber, Jr and D H. Tyras, *Chromatographia*, 28 (1989) 307
10 F A J van der Hoorn, F Boomsma, A J Man in't Veld and M. A D H Schalekamp, *J Chromatogr*, 487 (1989) 17
11 P. Hjemdahl, *Acta Physiol Scand, Suppl.*, 527 (1984) 43
12 A Mitsui, H Nohta and Y Ohkura, *J Chromatogr*, 344 (1985) 64
13 M. N H Irving and R. M Parkins, *J. Inorg Nucl Chem.*, 27 (1965) 270
14 P Hušek and J. Malikova, *Biogenic Amines*, submitted for publication
15 F. Smedes, J. C. Kraak and H. Poppe, *J Chromatogr.*, 231 (1982) 25