ELSEVIER

Journal of Chromatography B, 658 (1994) 63–68

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

High-performance liquid chromatographic determination of catecholamine metabolites and 5-hydroxyindoleacetic acid in human urine using a mixed-mode column and an eight-channel electrode electrochemical detector

Fumiko Mashige[a,*], Akiyuki Ohkubo[a], Yoshikazu Matsushima[b], Maiko Takano[b], Etsuko Tsuchiya[b], Hideko Kanazawa[b], Yoshiko Nagata[b], Nobuharu Takai[c], Noriko Shinozuka[d], Ichiro Sakuma[e]

[a]*Department of Laboratory Medicine, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan*
[b]*Kyoritsu College of Pharmacy, Shibakoen 1-5-30, Minato-ku, Tokyo 105, Japan*
[c]*Tokyo Medical College, 6-1-1, Shinjuku, Shinjuku-ku, Tokyo 160, Japan*
[d]*Institute of Industrial Science, University of Tokyo, 7-22-1, Roppongi, Minatoku, Tokyo 106, Japan*
[e]*Faculty of Science and Engineering, Tokyo Denki University, Ishizaka, Hatoyama-cho, Hiki-gun, Saitama 350-03, Japan*

First received 20 December 1993; revised manuscript received 22 April 1994

Abstract

An HPLC system for the simultaneous determination of acidic catecholamine metabolites, related compounds and 5-hydroxyindoleacetic acid (5-HIAA) in human urine was developed. A mixed-mode (C_{18}/anion-exchange) column with isocratic elution using citrate buffer and an eight-channel electrochemical detector were used. Vanilmandelic acid (VMA), 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenyllactic acid (vanillactic acid, VLA), homovanillic acid (HVA), vanillic acid (VA) and 5-HIAA in urine were determined simultaneously. Detection limits and inter ($n = 5$) and intra-assay ($n = 5$) coefficients of variation were satisfactory. The mean of analytical recoveries ($n = 3$, ±C.V. (%)) were between 97 ± 3.2 (VMA) and 105 ± 4.8 (VA). Correlations between the analytical results for VMA, HVA and 5-HIAA obtained by an established method and the present method were satisfactory. The mean ±2 S.D. of the excretion rates of VMA, DOPAC, VLA, HVA, 5-HIAA and VA in urine from healthy adult volunteers were 0.61–4.36, 0.13–1.02, 0–0.35, 0.67–6.55, 0.50–5.14 and 0–0.55 mg/g creatinine, respectively.

1. Introduction

The concentrations of catecholamines, serotonin, and their precursors and metabolites in body fluids are essential for diagnosis of neurological disorders. Analytical methods for these compounds have remarkably improved with the application of high-performance liquid chromatography (HPLC) [1] and are now widely used in combination with electrochemical detection (ED) [2–6]. However, simultaneous determination of these compounds in body fluids by

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00227-V

HPLC is difficult using single or dual ED without extraction procedures.

Matson *et al.* [7] have developed a gradient elution HPLC system with multichannel-ED and a three-dimensional display device for the simultaneous determination of these compounds. We have developed a new porous carbon coulometric electrode and an ED with four joint electrodes (multi-ED) in collaboration with Mitsubishi Petrochemical Co. (Kanagawa-ken, Japan) [8]. Using this multi-ED, we have elaborated a method for the simultaneous determination of neurotransmitters in cerebrospinal fluid [9]. This method was successfully applied to the determination of most metabolites of the catecholamines and serotonin in human urine. However VMA, could not be determined because it was oxidized irreversibly. With the reversed-phase column VMA and 3,4-dihydroxymandelic acid (DOMA) were not separated from interfering peaks in some urine samples.

In this report, we describe an HPLC method for the simultaneous determination of monoamine metabolites in urine. Oxidation currents were measured using an eight-channel ED by combining two multi-EDs. A mixed-mode C_{18}/anion-exchange) column was used. With this column, acidic metabolites were eluted slower compared with the conventional silica-ODS columns.

2. Experimental

2.1. Apparatus

The HPLC system (Fig. 1) consisted of a pump (LC-6A pump, Shimadzu, Kyoto, Japan), guard filter (Sumipax Filter PG-ODS, Sumika Analytical Center, Ohsaka, Japan), mixed-mode (C_{18}/anion-exchange) column (150 × 4.6 mm I.D., Alltech, Deerfield, IL, USA), a column oven (CTO-6A, Shimadzu), two multi-EDs (Mitsubishi Petrochemical, Kanagawaken, Japan) and a personal computer (NEC personal computer PC9801-DS, Nihon Denki, Tokyo, Japan). The electrodes of the coulometric multi-ED consisted of a working electrode made of porous carbon (3.0 mm diameter × 3.0 mm thickness), an Ag/AgCl reference electrode and a counter electrode [8].

2.2. Reagents

3,4-Dihydroxyphenylalanine (DOPA), dopamine (DA), epinephrine (E), norepinephrine (NE), DOPAC, 3-methoxytyramine (3-MT), HVA, VMA, VA, VLA, 4-hydroxy-3-methoxyphenylethyleneglycol (MHPG), 5-hydroxytryptophan (5-HTP) and 5-hydroxytryptamine (5-HT) were obtained from Sigma (St. Louis, MO, USA). DOMA, 3,4-dihydroxyphenylethyleneglycol (DOPEG), 5-HIAA, metanephrine (MN), normetanephrine (NMN) and 3,4-dihydroxyhydrocinnamic acid were obtained from Aldrich (Milwaukee, WI, USA). Other reagents were of HPLC grade and obtained from Wako Pure Chemical (Osaka, Japan).

2.3. Standards

Stock standard solutions were prepared at 1 g/l of DOMA, VMA, DOPAC, VLA, HVA, 5-HIAA, VA and 3,4-dihydroxyhydrocinnamic acid (internal standard, I.S.) in 0.1 mol/l perchloric acid, and were stored at −80°C. The

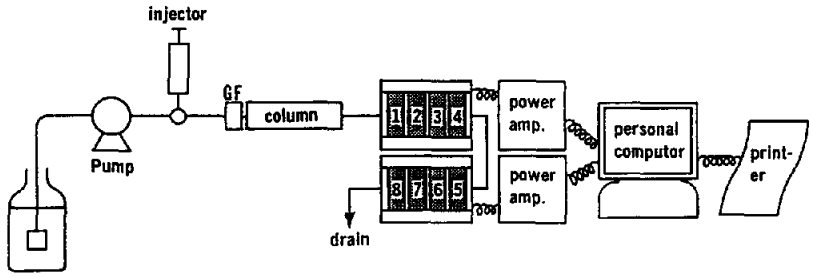

Fig. 1. Scheme of the HPLC system. GF: guard filter; 1–8: channel number of ED.

stock solutions were thawed immediately prior to use, mixed and diluted with mobile phase to 0.1 mg/l. These mixtures were used as working standard solutions and 100-μl aliquots were injected onto the HPLC system.

2.4. Mobile phase

The mobile phase was a mixture of 850 ml of 50 mmol/l citric acid in trisodium citrate buffer (pH 4.5) and 150 ml of methanol.

2.5. Samples

Urine samples were collected from healthy volunteers and patients and were immediately stored at −80°C until analysis. Before assay the samples were thawed and centrifuged at 1600 *g* at room temperature for 10 min. Fifty microliters of the supernatant, 50 μl of 3,4-dihydroxyhydrocinnamic acid (2 mg/l) and 900 μl of the mobile phase were mixed and a 100-μl volume was injected onto the HPLC system.

2.6. HPLC conditions

For the analysis of standard solutions and urine samples, the system illustrated in Fig. 1 was used. A 100-μl volume of sample was injected onto the HPLC. The flow-rate was 0.7 ml/min, and the temperature of the column was maintained at 35°C. The electroactive compounds were analyzed by the 8-channel ED (applied voltages: 200, 300, 350, 400, 450, 500, 550 and 600 mV in this order). The concentrations of the compounds were obtained from the ratio of their peak heights against that of the internal standard using a personal computing system [10]. The compounds and channel numbers used for their determination are; DOMA (1), VMA (4), DOPAC (1), VLA (5), HVA (5), 5-HIAA (2), VA (7) and I.S. (1).

2.7. Other methods

The concentration of creatinine in urine was determined by the Jaffé-reaction (rate-assay) using a Hitachi 736 auto-analyzer [11]. For a correlation test, VMA and HVA were also determined by HLC-726 VMA [12] (Tosoh, Tokyo, Japan), and 5-HIAA was also determined by HPLC-ED [13].

3. Results and discussion

3.1. Chromatography

The retention times of the compounds as a function of the pH of the citrate buffer (50 mmol/l) are shown in Fig. 2. Optimum separation was obtained at pH 4.5. To shorten the analysis time, 150 ml of methanol was added to 850 ml of the 50 mmol/l citrate buffer (pH 4.5). Voltammograms of the compounds are shown in Fig. 3. The voltages applied to the 8 channel electrodes were 200, 300, 350, 400, 450, 500, 550 and 600 mV, respectively.

Chromatograms of a standard solution are shown in Fig. 4. All analytes were well separated within 35 min. They were determined from the corresponding shaded peaks. Catecholamines, DOPA, NMN, MN, DOPEG, MHPG, 5-HT, 5-HTP and 3-MT were eluted within 5 min and were not separated from each other or interfering peaks. Chromatograms of a sample of human urine are shown in Fig. 5. The peak of DOMA at channel 1 was too small to allow exact estima-

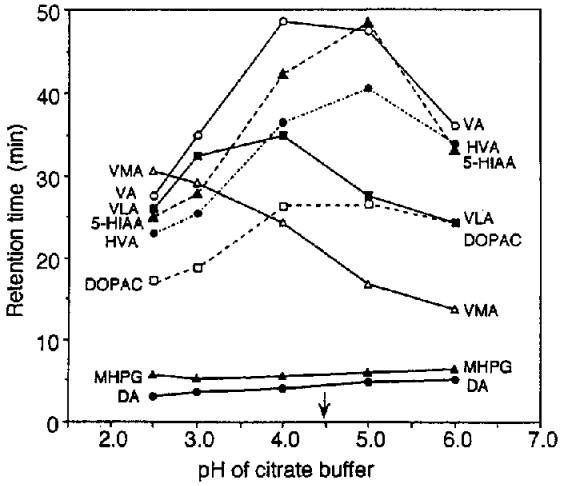

Fig. 2. Effect of pH of citrate buffer on retention times. HPLC conditions: flow-rate, 0.7 ml/min; column temperature, 35°C; mobile phase, 50 mmol/l citrate buffer containing 5% methanol.

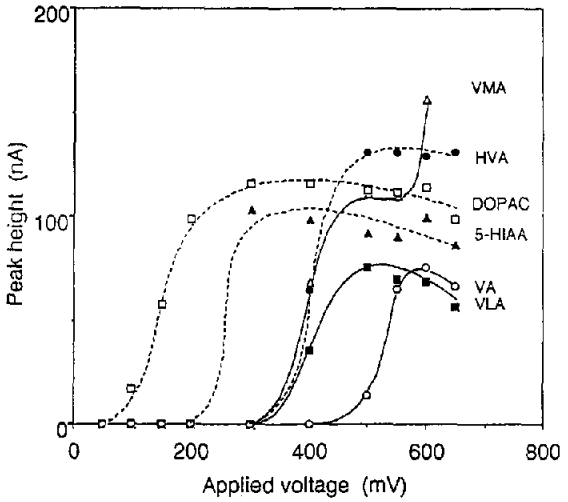

Fig. 3. Voltammograms of the multi-ED.

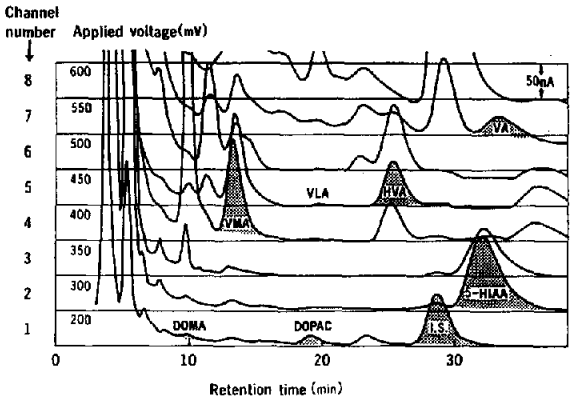

Fig. 5. Chromatograms of a human urine sample. The concentrations of DOMA, VMA, DOPAC, VLA, HVA, 5-HIAA and VA in this sample were 0.18, 5.26, 0.30, 0.26, 2.25, 3.35 and 0.82 mg/l, respectively.

tion. Large interfering peaks for DOMA appeared at channel 3 to 5 possibly at channel 2. Consequently, DOMA was not determined in the following experiments.

3.2. Analytical variables

The detection limits for the analytes at a signal-to-noise ratio of 3 were determined using standard solutions and urine samples. The lower limits (pg/100 μl) for standard solutions were 240 for VMA, 100 for DOPAC, 350 for VLA, 170 for HVA, 250 for 5-HIAA and 90 for VA.

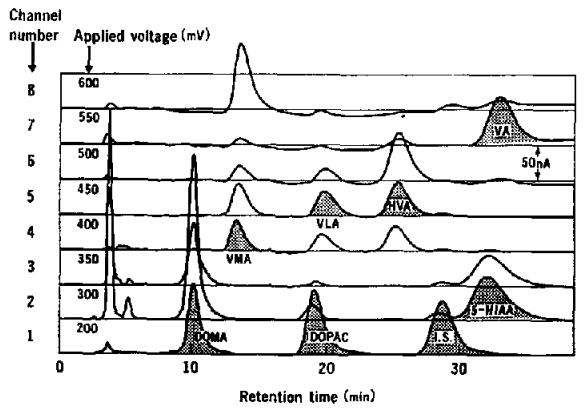

Fig. 4. Chromatograms of the standard solution. The shaded peaks were used for determination. The amount of each compound injected was 10 ng.

The corresponding values (mg/l) for the urine samples were 0.05 for VMA, 0.02 for DOPAC, 0.07 for VLA, 0.03 for HVA, 0.05 for 5-HIAA and 0.02 for VA.

The calibration curves for all compounds were linear within the range of 0.25 to 50 ng (Fig. 6).

Intra-assay precision was established from 5 assays of a patient urine and inter-assay precision on 5 consecutive days of the control urine which was prepared by adding 2 mg/l of standard solution to pooled urine. The mean values and coefficients of variation (C.V.) are shown in Table 1.

Five mg/l each of VMA and HVA, 1.0 mg/l each of DOPAC and 5-HIAA, and 0.5 mg/l VLA and VA were added to a patient urine. Analytical recoveries (mean $\pm$ C.V., $n = 3$) for VMA, HVA, DOPAC, 5-HIAA, VLA and VA were 97 ± 3.2, 100 ± 3.8, 105 ± 2.2, 104 ± 1.0, 100 ± 12.9 and 105 ± 4.8, respectively.

VMA and HVA in 66 urine samples were determined by HLC-726 VMA [12] (x) and the present method (y), and 5-HIAA in 37 urine samples by HPLC-ED [13] (x) and by the present method (y). Inter-assay C.V.s (mean, $n = 10$) of VMA, HVA and 5-HIAA by the reference methods were 2.6% (3.51 mg/l), 2.5% (3.50 mg/l) and 4.8% (2.20 mg/l), respectively. The correlation coefficient (r) and the equation of the regression lines for VMA, HVA and 5-HIAA

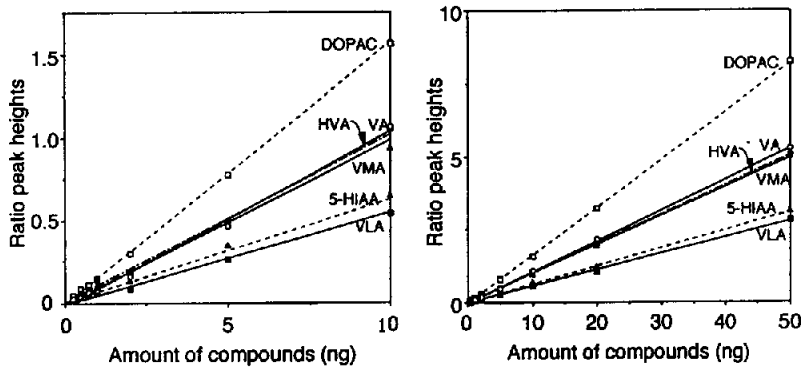

Fig. 6. Calibration graphs. (left) Range, 0.25–10 ng; (right) range, 2–50 ng.

were $r = 0.983$, $y = 1.01x + 0.27$, $r = 0.979$, $y = 0.92x + 0.20$ and $r = 0.993$, $y = 0.96x - 0.10$, respectively. Comparison of these results by the method of Eksborg [14] is shown in Fig. 7 (VMA, top; HVA, center; 5-HIAA, bottom). Good correlations were observed between the results at middle and high levels of VMA, HVA and 5-HIAA but not at lower levels.

3.3. Reference values

Urine samples were collected early in the morning from 20 healthy adult volunteers (10 males, 10 females, mean ± S.D. of age: 33.2 ± 11.8 years) who did not consume bananas, coffee and foods containing vanilla, such as ice cream, after 1 p.m. of the previous day. The amounts of compound excreted were expressed in mg of the compound per g of creatinine. Inter-assay C.V. ($n = 20$) for creatinine was 1.3% using a urine sample with 0.83 g/l of creatinine. The mean ± 2 S.D. of the excretion of VMA, DOPAC, VLA, HVA, 5-HIAA and VA in urine from the volunteers were 0.61–4.36, 0.13–1.02, 0–0.35, 0.67–6.55, 0.50–5.14 and 0–0.55 mg/g creatinine, respectively.

4. Conclusion

Diagnosis of neurological disorders is greatly aided when the levels of catecholamine metabolites and 5-HIAA in urine are known. These compounds can be simultaneously determined by HPLC-ED using a mixed-mode column which permitted the separation of VMA from interfering peaks.

The present method employs isocratic elution, which is superior to gradient elution in terms of simplicity, speed, reproducibility and reliability. Using the present method, we are studying the

Table 1
Precision

Compound	Inter-assay ($n = 5$)		Intra-assay ($n = 5$)	
	mean (mg/l)	C.V. (%)	mean (mg/l)	C.V. (%)
VMA	5.65	4.7	5.42	1.8
DOPAC	3.13	3.7	0.29	3.4
VLA	2.61	1.8	0.21	8.5
HVA	6.71	2.0	3.23	0.7
5-HIAA	4.95	4.2	4.31	0.9
VA	2.28	5.2	0.87	3.9

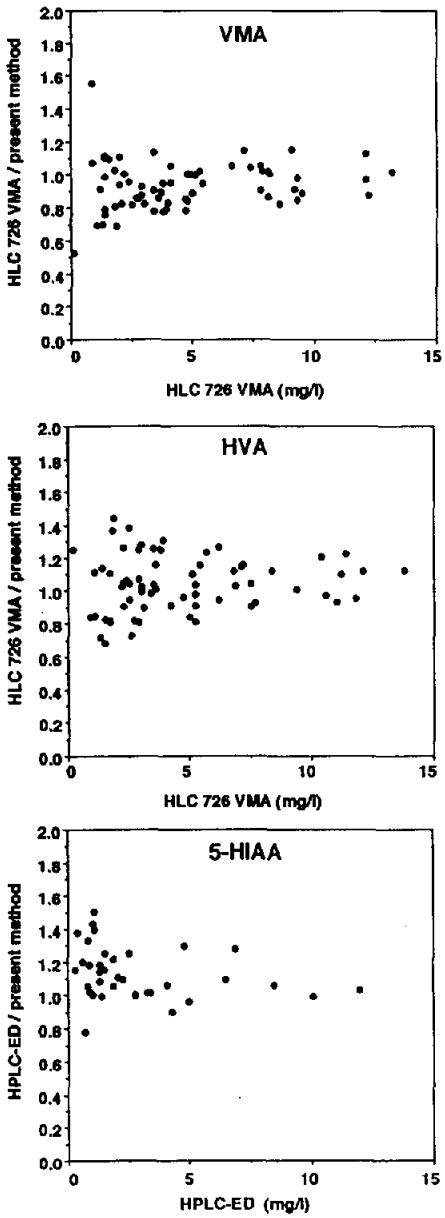

Fig. 7. Comparison between the values obtained by the present and the reference methods plotted according to ref. 14.

physiological variation of the metabolites of catecholamines and 5-HIAA in urine and reference values for people of different ages. The results will be reported shortly.

Acknowledgements

We like to thank Mitsubishi Petrochemical Co., Sumitomo Metal Bioscience Co. and Kurahashi-giken Co. for their support.

References

[1] B. Kågedal and D.S. Goldstein, *J. Chromatogr.*, 429 (1988) 177.
[2] A.M. Krstulovic, M. Zakaria, K. Lohse and L. Bertani-Dziedzic, *J. Chromatogr.*, 186 (1979) 733.
[3] C.G. Honegger, W. Krenger, H. Langemann and A. Kempf, *J. Chromatogr.*, 381 (1986) 249.
[4] P. Wester, J. Gottfries, K. Johansson, F. Klinteback and B. Winblad, *J. Chromatogr.*, 415 (1987) 261.
[5] E. Koyama, A. Minegishi and T. Ishizaki, *Clin. Chem.*, 34 (1988) 680.
[6] P.H. Gamache, M.L. Kingery and I.N. Acworth, *Clin. Chem.*, 39 (1993) 1825.
[7] W.R. Matson, P. Langlais, L. Volicer, P.H. Gamache, E. Bird and K.A. Mark, *Clin. Chem.*, 30 (1984) 1477.
[8] N. Takai, N. Shinozuka, F. Mashige, A. Ohkubo, N. Nukina, S. Iijima, Y. Fukui, I. Sakuma, A. Ito, Y. Matsushima, H. Kanazawa and Y. Nagata, in K. Miyai, T. Kanno and E. Ishikawa (Editors), *Progress in Clinical Biochemistry*, Elsevier, Amsterdam, 1992, p. 157.
[9] F. Mashige, N. Takai, N. Shinozuka, H. Wada, I. Sakuma, A. Ito, Y. Matsushima and A. Ohkubo, *Jpn. J. Clin. Chem.*, 22 (1993) 147.
[10] I. Sakuma, T. Dohi, H. Funakubo, N. Takai, A. Ohkubo, H. Niwa and A. Takeuchi, *Precision Machinery*, 1 (1986) 231.
[11] K. Larsen, *Clin. Chim. Acta*, 27 (1979) 158.
[12] S. Kawaguchi, N. Hirachi and M. Fukamachi, *J. Chromatogr.*, 567 (1991) 11.
[13] D.F. Davidson and J. Williamson, *Clin. Chem.*, 34 (1988) 768.
[14] S. Eksborg, *Clin. Chem.*, 27 (1981) 1311.