Journal of Chromatography, 145 (1978) 295—301
Biomedical Applications

CHROMBIO.124

Note

Application of paired-ion high-pressure liquid column chromatography to the analysis of L-3,4-dihydroxyphenylalanine metabolites

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(Received July 27th, 1977)

Since their discovery in mammals, catecholamines and their metabolites have posed a considerable challenge to the analytical chemist with respect to both their chromatography and quantitation. Of the various techniques developed to measure catechols in biological fluids and tissue [1, 2], most single analytical methods permit quantitative analysis of only a few compounds. Yet the diversity of catechol metabolism dictates development of analytical methods with greater scope, and only gas—liquid chromatography (GLC) with detection by flame ionization [3, 4], electron capture [4—7] or mass spectrometry [6—8] has allowed simultaneous assay of a broad range of metabolites. Volatilization for GLC requires derivatization, a process which for this group of compounds often demands careful, time-consuming preliminary purification, and is prone to error due to the lability of the products obtained [3—8]. With the advent of high-pressure liquid chromatography (HPLC), it was apparent that the method would lend itself to the resolution of a range of underivatized catechols and their metabolites with a speed and efficiency comparable to that of GLC. This has been borne out in a number of studies. Liquid chromatography of catechols, as mixtures of standards or in biological fluids and tissues, has been accomplished using ion exchange [9, 10], reversed-phase columns [11], as well as by ion-pair liquid—liquid partitioning [12]. We wish to report a variation of the HPLC technique which affords the separation of a range of catechols and their methyl ethers on octadecylsilica reversed-phase columns by paired-ion chromatogrpahy [13]. Pretreatment on alumina, which permits fractionation of catechols from non-catechols [14], was not necessary for standard mixtures but facilitated urinary analyses by this method. Using UV spectrophotometric detection the technique proved to be a simple, rapid means of quantitative analysis of L-3,4-dihydroxyphenylalanine (L-DOPA) and its major metabolites in glusulase-hydrolyzed urine of L-DOPA-treated parkinsonian patients.

MATERIALS AND METHODS

All catechols and their methyl ethers were purchased from Sigma (St. Louis, Mo., U.S.A.) with the exception of 3,4-dihydroxyphenylpyruvate, which was synthesized from L-DOPA [15]. 1-Heptanesulfonic acid (HSA) was obtained from Fischer Scientific (St. Louis, Mo., U.S.A.) or Waters Assoc. (Milford, Mass., U.S.A.) whereas alumina (neutral, EM Reagents) was purchased from Brinkmann Instruments (Des Plaines, Ill., U.S.A.). Glusulase is a product of Calbiochem (San Diego, Calif., U.S.A.). All other chemicals and solvents were reagent grade quality. All solvents were redistilled and, if used in the HPLC system, were passed through a 0.2-μm filter.

High-pressure liquid chromatography

The Waters Assoc. HPLC system used included a Model 6000A solvent delivery system, a Model U6K loop injector and a Model 440 absorbance detector which was operated at 280 nm. The column, also from Waters Assoc., was a 30 cm × 3.9 mm μBondapak C_{18} column (octadecylsilane chemically bonded to a silica solid support, 10-μm particle size range). All chromatograms were obtained at ambient temperatures.

Urinary analysis

Urine specimens (24 h) were collected from six male parkinsonian patients on Sinemet (0.3—0.8 g of L-DOPA and 30—80 mg of carbidopa, a peripheral decarboxylase inhibitor, per day), two female and one male parkinsonian patients on l-DOPA alone (1.5—4.0 g of L-DOPA per day) and two male parkinsonian patients who were neither receiving L-DOPA nor Sinemet. Control urine samples were obtained from five male laboratory workers. Urine was collected in specimen bottles which contained either 5 ml of 6 *M* hydrochloric acid or 10 g of sodium metabisulfite and were immediately stored at —20°.

To 1—3-ml aliquots of urine were added 0.1 volumes of 0.1 *M* sodium acetate buffer containing EDTA and ascorbate both at a final concentration of 3 m*M*. This solution, which was adjusted to a pH of 5.2, was incubated with 0.2 ml of glusulase (1.34 I.U. of glucuronidase and 2.07 I.U. of sulfatase) for 20 h at 37°. The sample was then made 0.4 *M* in perchloric acid and sodium metabisulfite was added to a final concentration of 50 m*M*. After shaking for 1 min, the mixture was centrifuged at 13,000 rpm for 15 min in a Sorvall SM-24 rotor at 4°. Upon cooling the supernatant to 0° in an ice bucket, 3 *M* potassium carbonate was added to bring its pH to 5. It was then centrifuged at 5000 rpm for 5 min at 4°. To the supernatant were added 100—300 mg of alumina (depending upon the initial volume of urine), 50 mg of sodium metabisulfite, and 1 ml of 0.15 *M* EDTA. After adjusting the pH of the slurry to 8.6 with 1 *M* sodium hydroxide, it was shaken for 3 min. The slurry was then placed onto a column (disposable Pasteur pipet) of 100—300 mg of alumina which had been pre-equilibrated with 0.2 *M* sodium acetate buffer, pH 8.6, containing 3 m*M* EDTA. After washing the column with 4 ml of the same buffer to remove non-catechols, the catechols were eluted with a 1 *M* hydrochloric acid solution containing 0.05 *M* sodium metabisulfite. The non-catechol and catechol fractions were dried and taken up in 1% aqueous acetic acid and passed through a 0.2-μm filter for HPLC analysis.

Data treatment

Metabolite levels were determined by peak height analysis using authentic compounds for comparison. Recovery of [^{3}H] dopamine (^{3}H-DA) from the alumina—HPLC method was 59.6 ± 1.3% in seven determinations. Using non-radioactive authentic standards the following recovery values were estimated: DA, 58%; DOPA, 55%; 3,4-dihydroxyphenylacetic acid (DOPAC), 76%; homovanillic acid (HVA), 83%; vanillyllactic acid (VLA), 62% and 3-methoxy-4-hydroxyphenylalanine (MDOPA), 41%.

RESULTS AND DISCUSSION

Elution profiles of a mixture of L-DOPA metabolites in the absence and presence of HSA are illustrated in Fig. 1. The change in the elution pattern reflects the interaction of the counter-ion with amino groups of the catechol derivatives to form hydrophobic ion pairs. Both the loss of charge and the introduction of the heptane group of HSA enhance the retention of the ion pair on the non-polar stationary phase (C_{18} hydrocarbon) of the μBondapak column, thereby increasing retention times of compounds with amino groups. For the group of catecholamines, amino acids and methyl ethers examined the retardation effect increased with retention time. As seen in Fig. 1, metab-

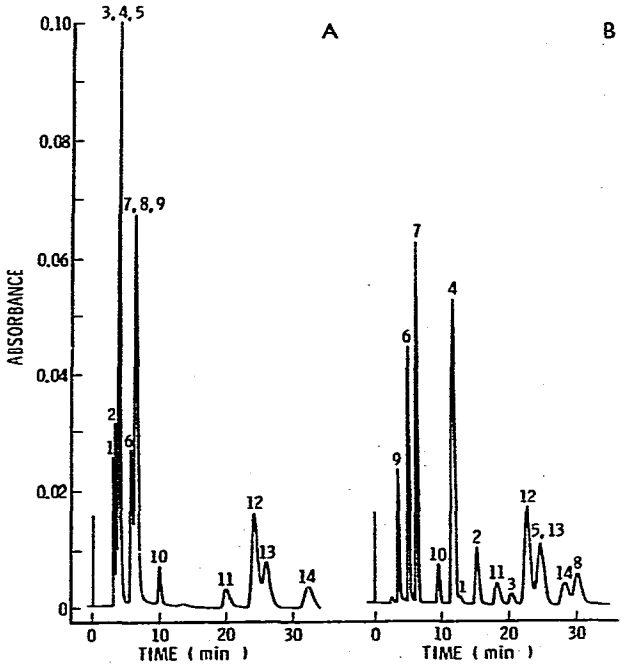

Fig. 1. Chromatogram of a standard mixture of L-DOPA metabolites in the absence (A) and presence (B) of 5 m*M* HSA. Column, μBondapak; mobile phase, water—methanol—acetic acid (95:5:0.95); flow-rate, 1.0 ml/min; inlet pressure, ca. 1000 p.s.i. Peaks: 1, norepinephrine; 2, epinephrine; 3, normetanephrine; 4, DOPA; 5, DA; 6, vanillylmandelic acid; 7, 3-methoxy-4-hydroxyphenylglycol; 8, MDOPA; 9, DHPP; 10, DOPAC; 11, VLA; 12, HVA; 13, vanillic acid (VA); 14, isovanillic acid (3-hydroxy-4-methoxy-benzoic acid). PHP is not shown; if present it would have had retention times of ca. 7 and 4 min in A and B, respectively.

TABLE I

URINARY LEVELS (MEAN ± S.E.) OF L-DOPA METABOLITES IN CONTROLS AND PARKINSONIAN PATIENTS

Urine samples were collected from controls and parkinsonian patients on drug regimens described in *Urinary analysis*. ND=not detectable.

Drug regimen	Number of subjects	Excretion (mg/24 h)						
		DOPA	DA	VLA	MDOPA	DOPAC	HVA	VA
Controls	5	1.51±0.61	0.98± 0.2	ND	ND	3.2 ±0.11	9.36± 1.0	3.3±1.06**
Sinemet	6	25.7 ±2.2	15.2 ± 3.3	23 ±6.2*	35.8±6.1	18.7 ±1.7	54.9 ± 8.6	10 ±3.5
L-DOPA	3	11 ±2.3	204 ±66	6.25±2.4*	129.2±7.8	623 ±30	774 ±91	4.6±1.8
None	2	0.39±0.11	0.66± 0.18	ND	ND	0.98± 0.02	8.1 ± 3.9	3.9±0.3

*$P < 0.1$.

**VA levels were measured on only three of the controls.

olites which do not contain amino groups were only slightly affected by addition of HSA. Two exceptions were *p*-hydroxyphenylpyruvate and 3,4-dihydroxyphenylpyruvate (DHPP) (Fig. 1), both of which exhibited two major peaks in all chromatograms. In both cases only the peak which had the shorter retention time exhibited a decrease in retention time when HSA was included in the mobile phase (Δt=2.8 and 2.6 min, respectively, in the system described in Fig. 1). The height of this same peak increased in the presence of HSA at the expense of the second peak (at 13 min in Fig. 1A). Since these phenomena were only observed with the pyruvates, it appeared that they might be attributable to keto—enol tautomerism. If so, the peak with the short retention time, which was more polar and predominated in the presence of 5 m*M* sodium borate, should be the enolate form. By measuring the change in absorbance of 330 nm it was ascertained that borate but not HSA shifts the equilibrium toward the enolate form [16]. Also HSA did not affect the pH of the mobile phase. Hence, a reason for the appreciable change in retention time of the pyruvates attributable to HSA is not apparent.

Despite the resolution obtained with standards, it was clear that given their complexity even hydrolyzed urine samples would require a prefractionation of free catechols from non-catechols by alumina chromatography [14]. As seen in Fig. 2, the combination of alumina and paired-ion reversed-phase HPLC allowed good resolution of the detectable metabolites present in a glusulase-hydrolyzed urine specimen from a Sinemet-treated patient. Using this technique urinary analyses were conducted for parkinsonian patients on L-DOPA

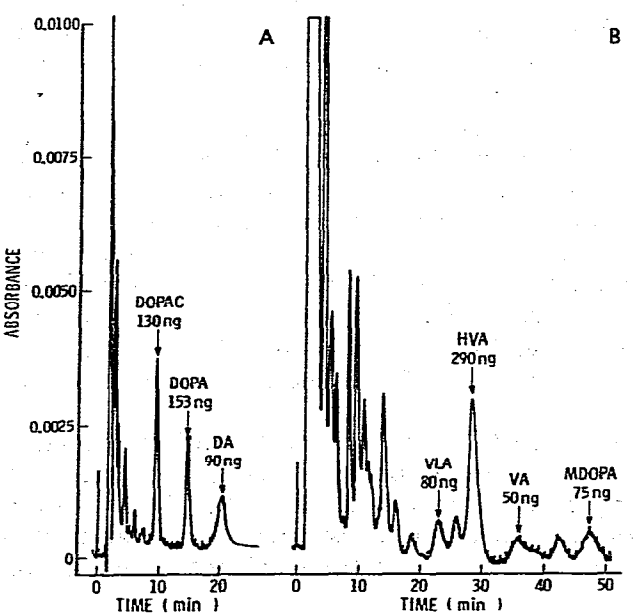

Fig. 2. Chromatogram of catechol (A) and non-catechol (B) fractions from a urine extract. Column, μBondapak; mobile phase, water—acetic acid (99:1) with 5 m*M* HSA; flow-rate, 1.5 ml/min; inlet pressure, ca. 1000 p.s.i. Results from a 3-ml urine sample from a parkinsonian patient on 0.44 g Sinemet per day.

or Sinemet as well as controls. Peak height measurements afforded values (Table I) which were comparable to amounts reported previously for urinary metabolites of parkinsonian patients on L-DOPA with or without a peripheral decarboxylase inhibitor taking into consideration variations in dosage [3, 17—19]. The changes in dopamine and vanillyllactic acid levels produced by the introduction of carbidopa confirmed the report of Sandler et al. [19] that this peripheral decarboxylase inhibitor shunts L-DOPA catabolism from decarboxylation to transamination.

The high molar absorptivity of catechol derivatives affords a sensitivity in the 10-ng range with the UV detector monitoring at 280 nm. Thus concentrations of most catechol metabolites in controls and non-treated parkinsonian patients were near the minimal level of detection (0.1 μg/ml of urine). The fact that neither DHPP nor 3-methoxy-4-hydroxyphenylpyruvate were detected in patients or controls under conditions in which added standards were recovered in 60% yields establishes that these compounds are not major urinary metabolites even when large doses of L-DOPA are administered.

The method described is a rapid, simple technique for analysis of the major metabolites of L-DOPA in urine of parkinsonian patients on L-DOPA chemotherapy. It would also be useful in other neurologic disorders in which L-DOPA metabolism is excessive [9]. Its adaptation to routine screening of normal urine, serum, and tissue samples awaits development of a detector with a sensitivity comparable to that of electron capture or mass fragmentography. Kissinger and co-workers [20—22] have demonstrated the excellent response of an electrochemical detector which is compatible with solvents used on reversed-phase columns [21]. It has proven useful for the measurement of hydrolyzed catechol levels (10—700 μg per 24 h) in normal unloaded urines [10] as well as in serum and brain [22, 23]. Although the electrochemical detector is more sensitive than UV spectrophotometry, it requires special sample pretreatment to eliminate electroactive contaminants and variable detector potential settings depending upon the types of catechol derivatives present [20—23]. Fluorimetric detectors have been used with HPLC instruments although in this case one must resort to derivatization with a fluorescent reagent. Fluorescamine has been reacted with the amino group of catecholamines, norepinephrine and dopamine, to yield a fluorescent derivative detectable at a lower limit of about 10 ng using HPLC with a fluorimetric detector [24, 25]. Optimal conditions for dansylation of the same catecholamines and separation of their dansylated derivatives by HPLC have also been reported [26].

A major advantage of HPLC over GLC is the ability to collect the eluate and analyze it further by more sensitive methods such as mass spectrometry. It has also been possible to study hepatic metabolism of DL-[7-^{3}H] norepinephrine by adding carrier metabolites to tissue homogenates, separating them by the HPLC technique described above, collecting the fractions and subjecting them to radioassay [27].

ACKNOWLEDGEMENT

This research was supported by NIH grant NS 12342. We would like to thank Dr. W.J. Burke for his assistance in obtaining patients for this study.

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