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Note

Determination of DOPA, dopamine, and 5-S-cysteinyl-DOPA in plasma, urine, and tissue samples by high-performance liquid chromatography with electrochemical detection

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There are two basic classes of melanin pigments in animals, i.e. dark eumelanin and reddish pheomelanin [1]. In melanocytes, tyrosinase converts tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and then to a highly reactive dopaquinone. In the absence of sulphydryl compounds, dopaquinone is converted to eumelanin in a complex series of reactions. If dopaquinone encounters cysteine (or glutathione), cysteinyl-DOPA isomers are produced among which 5-S-cysteinyl-DOPA is the major one [2]. Further oxidation of cysteinyl-DOPA gives rise to pheomelanin.

A high level of 5-S-cysteinyl-DOPA was first detected in melanoma tissues [3] and then in urine of melanoma patients [3]. The urinary excretion of this catecholic amino acid was found to reflect the degree of dissemination of melanoma [4] and is now routinely evaluated in many clinical laboratories. Although a fluorimetric method was originally developed for the determination of 5-S-cysteinyl-DOPA, a more sensitive and selective method was then reported using high-performance liquid chromatography (HPLC) with electrochemical detection [5—7].

Commonly, alumina extraction has been applied to extract catechols from biological samples before chromatographic analysis. However, the previously reported methods for 5-S-cysteinyl-DOPA determination use a batch procedure [5] or a column procedure [7] which is tedious and requires large amounts of

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samples, e.g. 6 ml of serum [5]. We have been studying the correlation between the level of DOPA and 5-S-cysteinyl-DOPA, the pigment precursors, and the type of melanogenesis [8,9]. In these studies, we found it convenient to develop a micro-batch procedure for catechol extraction which can be applied to plasma, urine, and tissue samples with minimum variations in the procedure and requires minimum amounts of samples. We describe here an improved method for the determination of DOPA, dopamine, and 5-S-cysteinyl-DOPA in biological samples by HPLC with electrochemical detection. Dopamine was included as an analyte because it is an immediate metabolite of DOPA in the catecholamine pathway.

EXPERIMENTAL

Chemicals

L-DOPA, dopamine hydrochloride, and 2-methyl-3-(3,4-dihydroxyphenyl)-DL-alanine (α-methyl-DOPA) were purchased from Sigma (St. Louis, MO, U.S.A.). Aluminum oxide, from Merck (Darmstadt, F.R.G.), was purified according to the method of Anton and Sayre [10]. 5-S-Cysteinyl-DOPA was prepared as described by us [11]. All other chemicals were of analytical grade and obtained from Wako (Osaka, Japan). Milli-Q system (Millipore, Bedford, MA, U.S.A.) ultrapure water was used throughout this study.

Chromatography

A Yanaco Model L-2000 liquid chromatograph (Yanagimoto, Kyoto, Japan) was used with a Yanaco Model VMD-101 electrochemical detector. The detector was set at +750 mV versus an Ag/AgCl reference electrode. Sensitivity was 4 and 32 nA at full scale. Separation was achieved on a C_{18} reversed-phase column (Yanaco ODS-A, particle size 7 μm, 250 × 4.6 mm) at 40°C. The mobile phase contained 10 g/l phosphoric acid and 7 g/l methanesulphonic acid in water, the pH being adjusted to 2.35 with 5 mol/l sodium hydroxide. To this, Na_2EDTA was added to a final concentration of 0.1 mmol/l. The flow-rate was 0.7 ml/min.

Samples

Blood samples were collected in centrifuge tubes containing Na_2EDTA and plasma was obtained by centrifugation. Urine samples (24-h) were collected at room temperature in bottles containing 50 ml of acetic acid and 1 g of sodium metabisulphite. Tissue samples were extracted with 0.4 mol/l perchloric acid [8]. All samples were stored frozen at −30°C until analysis.

Alumina extraction of DOPA, dopamine, and 5-S-cysteinyl-DOPA

In a conical plastic tube (1.5-ml volume) were placed 50 mg of acid-washed alumina, 100 μl of 2% (w/v) sodium metabisulphite, and 10 μl of 10 μg/ml α-methyl-DOPA in 0.1 mol/l hydrochloric acid as an internal standard. Either 1.0 ml of plasma, 100 μl of urine or tissue extract, or 10 μl of a standard solution containing 1 μg/ml each of DOPA, dopamine, and 5-S-cysteinyl-DOPA in 0.1 mol/l hydrochloric acid were added to the tube. For urine samples, 100 μl of 0.1 mol/l hydrochloric acid were added, and the mixture was extracted twice with

1 ml of ethyl acetate by shaking for 2 min on a JASCO MT-30 multi-tube mixer, and the ethyl acetate layer discarded by aspiration. Catechols were adsorbed onto alumina by adding 1.5 mol/l Tris—HCl buffer containing 2% (w/v) Na_2EDTA (pH 8.6) and immediately shaking for 5 min on the mixer. Volumes of the Tris buffer added were 0.3 ml for plasma and 1.0 ml for urine, tissue extract, and standard. For the analysis of tissue samples except for melanogenic tissues such as melanoma and hair, we used 0.3 ml of extract and 1.0 ml of 1.5 mol/l Tris—HCl buffer containing 2% (w/v) Na_2EDTA (pH 8.8). After centrifugation, alumina was washed twice with about 1.5 ml of water. Catechols were then eluted with 150 μl of 0.4 mol/l perchloric acid by shaking for 2 min. After centrifugation, 50—100 μl of the supernatant were injected into the HPLC system. Catechols were quantified from peak height ratios between catechols and α-methyl-DOPA for sample and standard.

RESULTS

Determination of DOPA, dopamine, and 5-S-cysteinyl-DOPA in biological samples

Fig. 1 shows typical chromatograms of urine samples from a normal subject and from a melanoma patient, and of a plasma sample from a normal subject, and of an extract of a B16 mouse melanoma. DOPA, dopamine, 5-S-cysteinyl-DOPA, and α-methyl-DOPA appeared at about 10, 11, 15.5, and 21 min, respectively. No large peaks appeared after α-methyl-DOPA. Although no interfering peaks were found in plasma and tissue samples after alumina extraction, urine samples contained multiple peaks which interfered with those of the catechols to be analysed. However, most of these peaks were eliminated by the extraction with ethyl acetate.

Method evaluation

The standard curves were linear over the range 0.5—100 ng of DOPA, dopamine, and 5-S-cysteinyl-DOPA after alumina extraction, when compared to 100 ng of the internal standard α-methyl-DOPA.

The absolute recoveries of catechols were estimated by comparison of the alumina-extracted standards with directly injected standards. The results, summarized in Table I, showed modest to good recovery and precision of the extraction procedure. The use of 0.4 mol/l perchloric acid was essential for the good recovery of 5-S-cysteinyl-DOPA; the recovery decreased to 54.3% with 0.2 mol/l perchloric acid. Similar results have been reported by Hansson et al. [6].

The analytical recoveries of the catechols added to normal plasma and urine samples were satisfactory, as indicated in Table II. Since catechols, especially 5-S-cysteinyl-DOPA, are susceptible to autoxidation, great care should be taken to shake the mixture immediately after the addition of Tris—HCl buffer.

Estimation of DOPA and 5-S-cysteinyl-DOPA in plasma

Plasma samples from 31 normal subjects were analysed. Plasama concentrations (mean ± S.D.) of DOPA and 5-S-cysteinyl-DOPA were 0.85 ± 0.38 ng/ml and 0.37 ± 0.30 ng/ml, respectively. These values are much lower than those

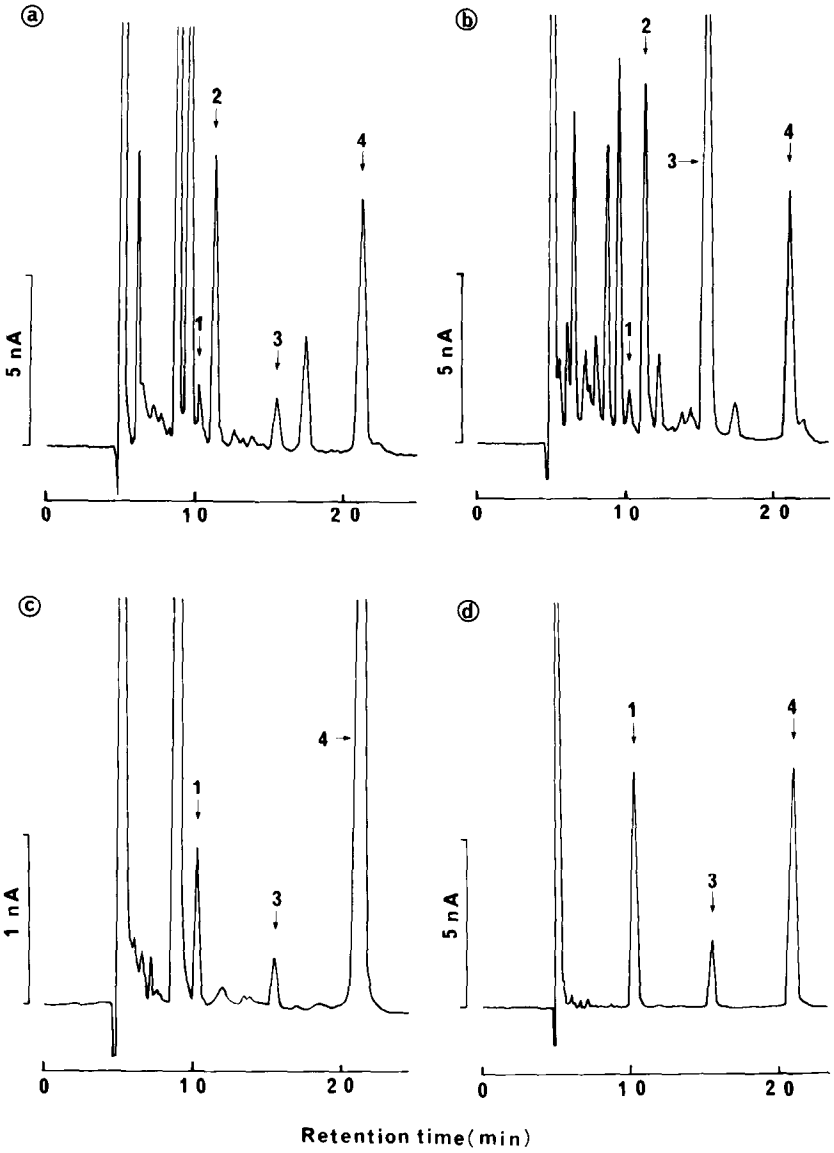

Fig. 1. Chromatograms of (a) a urine sample from a normal subject, (b) a urine sample from a melanoma patient, (c) a plasma sample from a normal subject, and (d) an extract of a B16 mouse melanoma. Chromatographic conditions: column, Yanaco ODS-A (7 μm particle size); mobile phase, 10 g/l phosphoric acid and 7 g/l methanesulphonic acid, pH being adjusted to 2.35 with 5 mol/l sodium hydroxide, Na_2EDTA being added to a final concentration of 0.1 mmol/l; flow-rate, 0.7 ml/min; temperature, 40°C; detector, +750 mV versus Ag/AgCl, 4 and 32 nA at full scale. Peaks: 1 = DOPA, 2 = dopamine, 3 = 5-S-cysteinyl-DOPA, 4 = α-methyl-DOPA.

reported by Hansson et al. [5]. This discrepancy may be ascribed to better clean-up and separation of the present procedure. For plasma samples, the detection limits at a signal-to-noise ratio of 2 were about 0.02 ng/ml for DOPA and dopamine and about 0.05 ng/ml for 5-S-cysteinyl-DOPA (injection volume 100 μl). Dopamine was below the detection limit.

TABLE I

ABSOLUTE RECOVERIES OF DOPA, DOPAMINE, AND 5-S-CYSTEINYL-DOPA AFTER ALUMINA EXTRACTION

Mean ± S.D. for 5 determinations.

Compound	Amount (ng)	Recovery (%)	C.V. (%)
DOPA	10	76.4 ± 1.4	1.8
Dopamine	10	75.4 ± 1.3	1.7
5-S-Cysteinyl-DOPA	10	69.6 ± 3.7	5.3
α-Methyl-DOPA	100	74.9 ± 1.5	2.0

TABLE II

ANALYTICAL RECOVERIES OF DOPA, DOPAMINE AND 5-S-CYSTEINYL-DOPA ADDED TO NORMAL PLASMA AND URINE SAMPLES

Mean ± S.D. for 5 determinations.

Sample	Amount added (ng)	Recovery (%)		
		DOPA	Dopamine	5-S-Cysteinyl-DOPA
Plasma	10	94.7 ± 4.2	104.1 ± 4.9	92.2 ± 2.3
Plasma	100	93.7 ± 0.5	105.9 ± 1.1	94.0 ± 0.9
Urine	100	99.7 ± 7.3	102.3 ± 6.7	99.5 ± 8.3

DISCUSSION

Previous studies by Rorsman and his associates [3,4,12,13], by us [8,9], and by others have shown that the determination of DOPA and 5-S-cysteinyl-DOPA in urine, blood, and tissue samples has a biochemical and clinical significance. HPLC with electrochemical detection has been adopted for the determination of these catecholic amino acids as for catecholamines.

Commonly, urinary 5-S-cysteinyl-DOPA is determined after alumina extraction. Kågedal and Pettersson [7] found that the alumina extraction alone was not enough to remove many interfering compounds and they recommended a combination of ion-exchange chromatography with alumina extraction for the clean-up. We replaced ion-exchange chromatography with ethyl acetate extraction to simplify the procedure.

DOPA and 5-S-cysteinyl-DOPA in serum have also been determined by HPLC with electrochemical detection after alumina extraction [5,6,13]. However, as far as we know, the reported method requires 6 ml of serum, which seems to be a burden to patients. Our improved method requires only 1 ml of plasma, and, moreover, involves only minimum variations in the procedure for plasma, urine, and tissue samples. This enabled us to analyse different types of samples at the same time.

Hansson et al. [6] described a good separation of DOPA, cysteinyl-DOPA isomers, and catecholamines using a mobile phase of pH 1.75. We also obtained similar results with a pH 1.75 buffer, but found a rapid deterioration of the column due to the low pH. At pH values higher than 2.50, uric acid, which

appears just in front of DOPA, comes too close to DOPA. Thus, we chose pH 2.35 for the mobile phase.

Advanced methodology has recently been applied to the determination of urinary 5-S-cysteinyl-DOPA. First, the use of a diastereomer of naturally occurring 5-S-L-cysteinyl-L-DOPA as an internal standard should improve the precision of the analysis because of the close similarity in chemical properties [14,15]. Secondly, phenylboronate affinity gel can be used to purify 5-S-cysteinyl-DOPA with a higher selectivity [15]. If only 5-S-cysteinyl-DOPA is to be analysed, the combined use of these two methodologies would be advantageous over the present method. However, our method can analyse not only 5-S-cysteinyl-DOPA but also DOPA and dopamine. Furthermore, our method is sufficiently simple, sensitive, precise, and accurate for routine use.

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