Journal of Chromatography, 272 (1983) 287—297
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1498

DETERMINATION OF URINARY 5-S-CYSTEINYLDOPA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received August 3rd, 1982)

SUMMARY

A high-performance ion-pair liquid chromatographic method with electrochemical detection is described, which is suitable for routine determination of urinary 5-S-cysteinyldopa. The clean-up procedure includes a first purification step on the cation exchanger AG 50 W (H^+). After desorption from the resin at moderately raised pH the catecholic amino acid is adsorbed on alumina at pH 8.6, washed and finally desorbed by elution with perchloric acid. By the combined clean-up procedures, easily oxidized compounds are eliminated, which otherwise cause a number of interfering peaks in the chromatography. The synthesis of 5-S-cysteinyl-L-3,4-dihydroxyphenyl [2,3-^{3}H]alanine is described, and this tritium-labelled 5-S-cysteinyldopa is used to determine the recovery in the sample. The precision (C.V. = 5.7% at low and C.V. = 4.9% at high 5-S-cysteinyldopa concentration) and recovery (105.0 ± 8.6%) were satisfactory. The mean urinary excretion was 0.34 ± 0.13 (S.D.) μmol per 24 h (range 0.02—0.58 μmol per 24 h) in healthy subjects (n = 24) and in patients with melanoma metastates (n = 13) the excretion ranged from 0.9 to 4.8 μmol per 24 h.

INTRODUCTION

Malignant melanoma is one of those carcinomas that is increasing most rapidly in white-skinned populations and causes a substantial number of deaths [1—3]. This cancer produces a number of substances which have been suggested as tumor markers [4—7]. As measured by a fluorimetric method [8] the urinary excretion of 5-S-cysteinyldopa was recently evaluated for that purpose and found to be a good estimate of the increased tumor burden in patients with metastases [9].

Recently a method with increased sensitivity and selectivity was described [10, 11] for the determination of 5-S-cysteinyldopa in urine. After precipitation of urinary proteins with perchloric acid, the sample, spiked with an

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internal standard, was treated with activated alumina which adsorbs the catechols at alkaline pH. After washing, 5-S-cysteinyldopa and other catechols were eluted with perchloric acid. The sample was then subjected to ion-pair high-performance liquid chromatography (HPLC) with electrochemical detection.

When we tried to apply and validate this method for routine clinical chemical determinations of urinary 5-S-cysteinyldopa, we found a number of drawbacks such as peaks interfering with the 5-S-cysteinyldopa peak and the occurrence of peaks with long retention times. The latter fact made the method unsuitable for routine determinations because of long chromatographic runs. Instability of the alumina-treated sample precluded storage until the next day. Furthermore HPLC with electrochemical detection gave a high baseline current and a variable detector response to 5-S-cysteinyldopa. Although we soon found that addition of disodium ethylenediaminetetraacetate (Na_2EDTA) both in the final sample eluate and in the moble phase improved the stability, the remaining drawbacks hampered the usability of the method in routine clinical work. We therefore conducted systematic studies, especially on the clean-up procedures for 5-S-cysteinyldopa. Further, some modifications of the HPLC procedure are reported.

EXPERIMENTAL

Chemicals

Tyrosinase from mushroom and cysteine as free base were obtained from Sigma (St. Louis, MO, U.S.A.) and 3,4-dihydroxy-L-phenylalanine (L-DOPA) was from Merck (Darmstadt, G.F.R.). L-3,4-Dihydroxyphenyl[2,3-^{3}H]alanine (specific activity 77.7 GBq/mmol) was obtained from The Radiochemical Centre (Amersham, Great Britain). Cation-exchange chromatography was performed with AG 50 W-X4, 200—400 mesh, and with AG 50 W-X8, 100—200 mesh, from Bio-Rad Labs. (Richmond, CA, U.S.A.). Aluminium oxide, Brockman activity II, from BDH Chemicals (Poole, Great Britain) was pretreated according to the procedure of Anton and Sayre [12].

Methanesulphonic acid (puriss.) was from Fluka (Buchs, Switzerland). Phosphoric acid (85%) and Na_2EDTA were from Merck and all other reagents were of reagent grade quality.

Synthesis of 5-S-cysteinyldopa

5-S-Cysteinyldopa was synthesized from cysteine and L-DOPA by the tyrosinase reaction according to the description of Agrup et al. [13]. They performed mass spectrometric analysis of the compound synthesized. The identity of the substance we obtained was checked by comparison of the retention time in HPLC with that obtained with an authentic sample of 5-S-cysteinyldopa obtained from these authors. Furthermore, we obtained similar R_F values on cellulose thin-layer chromatography and similar UV-absorption curves [13]. The concentration of a standard solution was determined with use of the molar extinction coefficient at 292 ($\log \epsilon = 3.47$) and 255 nm ($\log \epsilon = 3.58$) as given by Prota et al. [14], and checked by comparison with the total sulphur content determined by a wet combustion technique

[15] followed by sulphate determination [16]. Good agreement was obtained with these methods. Stock solution of 5-S-cysteinyldopa, 2.75 mmol/l, was prepared in hydrochloric acid, 2 mol/l. This stock solution of 5-S-cysteinyldopa was found to be stable for two years as it gave correct results when UV absorbance and HPLC peaks were compared with a newly synthesized preparation. The working standard solution (275 nmol/l) of 5-S-cysteinyldopa was prepared in perchloric acid, 1.0 mol/l, containing Na_2EDTA, 20 mmol/l.

Synthesis of 5-S-cysteinyl-L-3,4-dihydroxyphenyl[2,3-^{3}H]alanine

For synthesis of tritium-labelled 5-S-cysteinyldopa, 1000 µl of tritium labelled L-DOPA were mixed with 40 µl of a solution containing 0.55 mg of cysteine and 0.55 mg of L-DOPA in phosphate buffer, 0.5 mol/l, pH 6.5. The reaction was started by addition of 150 µg of tyrosinase dissolved in 20 µl of phosphate buffer. After 13 min the enzyme reaction was stopped by the addition of 120 µl of hydrochloric acid, 6.0 mol/l and 160 µl of sodium metabisulphite, 50 g/l. The product obtained was then transferred to a 180 × 10 mm column of AG 50 W-X4, 200—400 mesh, equilibrated with hydrochloric acid, 1.0 mol/l. After washing with 50 ml of hydrochloric acid, 1.0 mol/l, elution was performed with hydrochloric acid, 2.0 mol/l.

Urine collection

Urine was collected for 24 h in plastic bottles containing 5 ml of thymol, 0.7 mol/l in isopropanol, added as preservative [17], or containing 50 ml of glacial acetic acid and 1 g of sodium metabisulphite [9], and stored at −18°C until processed.

Procedure for purification of urinary 5-S-cysteinyldopa

To 6 ml of urine, 50 µl of tritium-labelled 5-S-cysteinyldopa and 2 ml of Na_2EDTA, 0.27 mol/l, were added followed by 0.8 ml of perchloric acid, 4 mol/l. After mixing, the precipitate was removed by centrifugation, and 0.1 ml was taken off for radioactivity determination. From the supernatant 4 ml were transferred to a 4.0 × 0.5 cm column (Econo-Column, Bio-Rad Labs.) of AG-50 W-X8 (100—200 mesh) which was equilibrated with hydrochloric acid, 1.0 mol/l. After washing with 2 ml of hydrochloric acid, 1 mol/l, elution was performed with citrate—phosphate buffer, 0.5 mol/l and pH 4.0 which was prepared by mixing 380 ml of citric acid, 0.5 mol/l, with 500 ml of disodium hydrogen phosphate, 0.5 mol/l. The first 3 ml of the eluate were discarded and the following 7 ml were transferred to a beaker containing 400 mg of activated alumina and 10 mg of sodium metabisulphite. Then the pH was raised by adding about 1.0 ml of sodium hydroxide, 4.0 mol/l, until pH 8.0 followed by final adjustment to pH 8.6 with sodium hydroxide, 0.4 mol/l. After stirring, the alumina was quantitatively transferred to a 100 × 20 mm I.D. separating funnel with glass filter (porosity 2), the liquid sucked off and the alumina washed four times with 10-ml portions of water. Desorption was effected by shaking for 15 min with 1.5 ml of perchloric acid, 1.0 mol/l containing Na_2EDTA, 20 mmol/l. The amount of 5-S-cysteinyldopa in the eluate was then determined by HPLC by comparison with standard solutions, and 0.1 ml was taken from the eluate for radioactivity counting and recovery determination.

High-performance liquid chromatography

We used a Constametric III pump from LDC (Riviera Beach, FL, U.S.A.) and a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 sample injector with a 100-μl sample loop. The electrochemical detector consisted of a TL-5A electrochemical cell from Bioanalytical Systems (West Lafayette, IN, U.S.A.). Working and auxiliary electrodes were made of glassy carbon. The detector was operated at +0.75 V vs. an Ag/AgCl reference electrode. The detector cell, placed in a Faraday cage to minimize electrical disturbances, was connected to an electrochemical controller Model LC-2A, also from Bioanalytical Systems.

The HPLC column was a Supelcosil LC-8 column (150 × 4.6 mm), particle size 5 μm, from Supelco (Bellefonte, PA, U.S.A.). In order to protect the analytical column, a precolumn packed with Solvecon Silica, particle size 37--53 μm (Pierce, Rockford, NJ, U.S.A.) was put in line between the pump and injector. The mobile phase was a water solution containing phosphoric acid, 30 mmol/l, methanesulphonic acid, 42 mmol/l, and Na_2EDTA, 0.1 mmol/l. The Na_2EDTA was dissolved in water before adding the other reagents, and final adjustment to pH 2.55 was obtained by adding sodium hydroxide, 5 mol/l, about 65 ml per 5000 ml final volume. The mobile phase was degassed by vigorous helium bubbling and, furthermore, a low helium flow-rate was maintained through the mobile phase bottle during chromatography.

Radioactivity determinations

From the supernatant obtained after perchloric acid precipitation 0.1 ml was transferred to a glass scintillation vial containing 10 ml of the liquid scintillator (Riafluor, New England Nuclear, Boston, MA, U.S.A.). Similarly the radioactivity was determined in a 0.1-ml aliquot from the final eluate. The β-liquid scintillation counter was an instrument from Intertechnique (Model SL-4001).

Calculations

The amount of 5-S-cysteinyldopa in 0.1 ml of the final eluate (A_E) was determined by comparing the sample peak height (H_{sample}) with the peak height ($H_{standard}$) of the standard solution (27.5 pmol per 100 μl) according to the formula

$$A_E = \frac{H_{sample}}{H_{standard}} \times 27.5 \text{ pmol}$$

The amount of 5-S-cysteinyldopa in 0.1 ml of the supernatant after perchloric acid precipitation (A_P) was determined according to the formula

$$A_P = A_E \times \frac{cpm_P}{cpm_E} \text{ pmol}$$

where cpm_P and cpm_E are the radioactivity counts in the supernatant and eluate, respectively.

The total amount of 5-S-cysteinyldopa in the mixed sample (volume 8.85 ml) is then $A_P \times 88.5$ pmol. From this, the added chemical amount of tritium-labelled 5-S-cysteinyldopa (493 pmol) was subtracted. The original concentration in the urinary sample was then finally determined as

$$\frac{(A_P \times 88.5) - 493}{6 \times 1000}\ \mu\text{mol/l}.$$

RESULTS

Synthesis of tritium-labelled 5-S-cysteinyldopa

5-S-Cysteinyl-L-3,4-dihydroxyphenyl[2,3-^{3}H]alanine was synthesized by scaling down the amounts and volumes used in the macroscale method for 5-S-cysteinyldopa synthesis. It should be noted that a number of side reaction products may be obtained, especially if the tyrosinase reaction is continued for too long. Fig. 1 shows the chromatography of a successful synthesis of tritium-labelled 5-S-cysteinyldopa on AG 50 W-X4. A major radioactivity peak was identified by HPLC as 5-S-cysteinyldopa. To check the radiochemical purity of the labelled 5-S-cysteinyldopa, samples from different parts of the radioactivity peak were analyzed by HPLC. The effluent from the liquid chromatograph was collected after the electrochemical cell, and the radioactivity was determined. Good agreement was obtained between the electrochemical detection and the radioactivity determinations, except for the first few fractions of the 5-S-cysteinyldopa peak (Fig. 1) which contained a small radioactive contaminant. Otherwise the fractions contained pure 5-S-cysteinyldopa, and after pooling fractions 55–73, 90 ml of tritium-labelled 5-S-cysteinyldopa, 9.86 μmol/l, were obtained with a specific activity of 188 mCi/mmol (7.0 GBq/mmol).

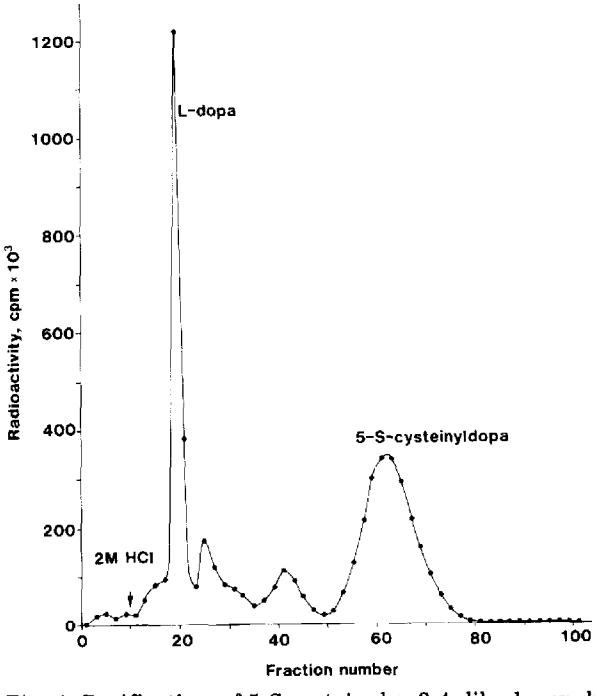

Fig. 1. Purification of 5-S-cysteinyl-L-3,4-dihydroxyphenyl[2,3-^{3}H]alanine synthesized from L-3,4-dihydroxyphenyl[2,3-^{3}H]alanine and cysteine by chromatographic cation exchange (AG 50 W-X4, bed dimensions 180 × 10 mm). Tritium activity was determined in aliquots from the fractions. The L-DOPA and 5-S-cysteinyldopa were identified by HPLC.

Optimization of HPLC conditions

We first conducted the HPLC in a manner similar to that described by Hansson et al. [10]. However, when analyzing urine samples we found that several other peaks with retention times up to 2 h were obtained. Various procedures were then tried in order to select a procedure which gave a well cleaned-up sample. Preliminary investigations with a combined treatment of the sample with a cation exchanger and alumina gave promising results and showed that the HPLC conditions could favourably be changed to give shorter retention times for 5-S-cysteinyldopa. Therefore, we changed to a Supelcosil LC-8 column which gives a shorter retention time and in order to spare the HPLC equipment the pH of the mobile phase was increased to 2.55, which also gives a lower capacity factor [11].

Initially we found high background currents (usually 5—22 nA) with the original composition of the mobile phase. The effects of including Na_2EDTA in the mobile phase were therefore studied and we found that a concentration of 0.1 mmol/l gave a satisfactory baseline current (<1 nA).

Purification of urinary 5-S-cysteinyldopa

In preliminary experiments using the strong cation exchanger AG 50 W-X8 the 5-S-cysteinyldopa was desorbed from the ion exchanger by elution with hydrochloric acid, 2 mol/l [13]. Although an improved clean-up of the sample was obtained, the 5-S-cysteinyldopa was eluted from the column in a large volume. However, it was found that also when 5-S-cysteinyldopa was eluted with citrate—phosphate buffer, 0.5 mol/l, pH 4.0, an improvement of the purification was obtained. An elution diagram of 5-S-cysteinyldopa from AG 50 W-X8 is shown in Fig. 2. It can be seen that most of the 5-S-cysteinyldopa could be collected in an eluate of 7 ml. Fig. 3 shows the HPLC results after purification of a urinary sample from a patient with malignant melanoma on AG 50 W-X8, and for comparison purposes Fig. 4 shows the results obtained when the clean-up was performed with alumina as in the original procedure. A number of peaks were obtained in both chromatograms and some of the peaks interfered with the 5-S-cysteinyldopa peak. However, when the two procedures were combined a marked improvement was found (Figs. 5 and 6). This combined clean-up procedure was therefore taken as the final procedure.

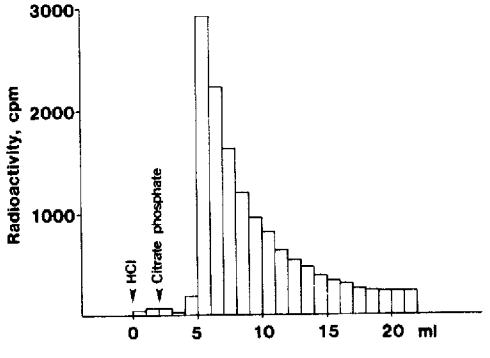

Fig. 2. Elution of 5-S-cysteinyl-L-3,4-dihydroxyphenyl[2,3-^{3}H]alanine from AG 50 W-X8 (100—200 mesh) with citrate—phosphate buffer, 0.5 mol/l, pH 4.0.

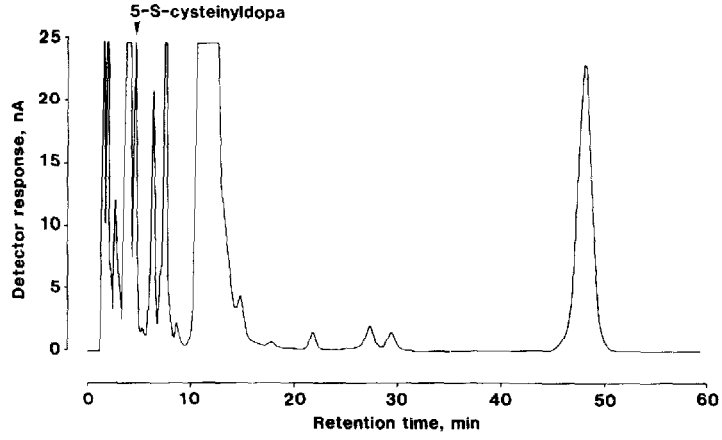

Fig. 3. HPLC of urine from a patient with malignant melanoma. The urine was pretreated on AG 50 W-X8 (cf. Fig. 2) and 7 ml of eluate were pooled.

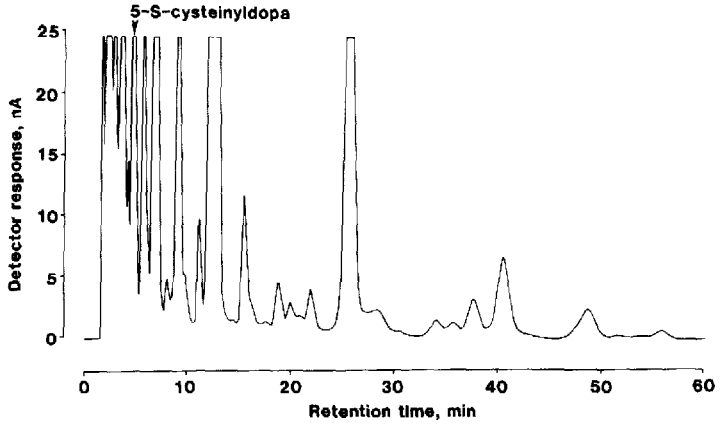

Fig. 4. HPLC of urine (same as in Fig. 3) adsorbed on alumina at pH 8.6 and desorbed with perchloric acid, 1.0 mol/l.

For the determination of urinary concentration the tritium-labelled 5-S-cysteinyldopa was added to the urine as described in the Experimental section.

Method evaluation

The standard curve was linear over the range 3.5–110 pmol of 5-S-cysteinyldopa injected, and no intercept was found. The precision of the HPLC was evaluated from peak height measurements after repeated injections (n = 10) of standard 5-S-cysteinyldopa (27.5 pmol per 100 μl) during 2 h. A coefficient of variation (C.V.) of 1.04% was found. When during ordinary working days the standard solution was injected with longer intervals the C.V. varied from 0.91 to 3.4%. This clearly showed that the determinations of 5-S-cysteinyldopa in the samples could well be done by comparison with an external 5-S-cysteinyldopa standard.

The intra-assay precision of the method for urinary determination was evaluated from duplicate analyses. For urines (n = 24) with 5-S-cysteinyldopa

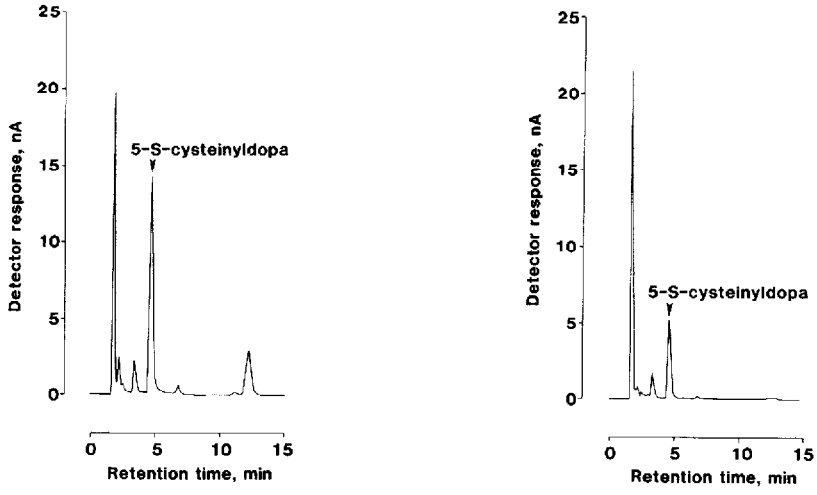

Fig. 5. HPLC of urine after combined treatment with AG 50 W-X8 and alumina. Same urine as in Figs. 3 and 4.

Fig. 6. HPLC of urine from a healthy subject. Urine clean-up as in Fig. 5.

concentrations between 0.02 and 0.6 μmol/l (mean 0.344 μmol/l) the standard deviation (S.D.) was calculated as 0.020 μmol/l which gave the C.V. as 5.7%. From the S.D. of 0.020 μmol/l the detection limit was calculated as 0.04 μmol/l. For urines with high 5-S-cysteinyldopa concentrations (n = 19) the resulting C.V. was 4.9% (mean = 2.60 μmol/l, S.D. = 0.128 μmol/l). The inter-assay variation was evaluated from repeated (n = 18) determination during a period of three months of a urinary sample stored frozen. From the mean of 0.521 μmol/l and an S.D. of 0.069 μmol/l the C.V. was found to be 13.3%. The recovery of 5-S-cysteinyldopa added to urines was satisfactory (Table I).

TABLE I

ANALYTICAL RECOVERY OF 5-S-CYSTEINYLDOPA ADDED TO SEVEN URINE SAMPLES, CORRESPONDING TO AN INCREASE IN CONCENTRATION OF 0.69 μmol/l

Initially present (μmol/l)	After addition (μmol/l)	Increase found (μmol/l)	Recovery (%)
0.24	0.97	0.73	105.8
0.50	1.27	0.77	111.6
0.29	1.02	0.73	105.8
0.44	1.20	0.76	110.1
0.14	0.91	0.77	111.6
0.24	0.95	0.71	102.9
0.49	1.09	0.60	87.0
	Mean ± S.D.		105.0 ± 8.6

Urinary excretion of 5-S-cysteinyldopa

The excretion of 5-S-cysteinyldopa was measured in 24 healthy subjects (12 men and 12 women) who collected urine during periods unexposed to sunshine. The mean (± S.D.) urinary excretion was 0.34 ± 0.13 μmol per 24 h (range 0.02—0.58 μmol per 24 h) and no sex difference was found (men 0.36 ± 0.13 μmol per 24 h, women 0.33 ± 0.13 μmol per 24 h). The distribution of the urinary excretion is illustrated in Fig. 7 together with the results from 13 patients with a diagnosis of malignant melanoma with metastases. The urinary excretion of 5-S-cysteinyldopa in the latter was highly increased, ranging from 0.9 to 4.8 μmol/l.

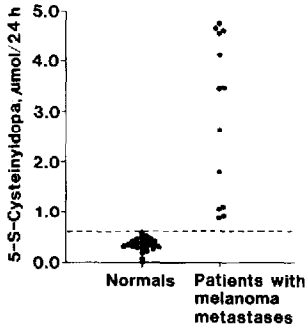

Fig. 7. Urinary excretion of 5-S-cysteinyldopa in healthy subjects and in patients with advanced malignant melanoma.

DISCUSSION

Classically catechols are purified from urine by alumina adsorption at alkaline pH and desorption with a strong mineral acid [12]. This method has also been used in the clean-up of urinary 5-S-cysteinyldopa [8, 10, 11], but in our hands did not give satisfactory results. However, since 5-S-cysteinyldopa in addition to the catechol moiety also contains an amino acid residue, we tried to use a clean-up procedure involving a strong cation exchanger. Also this procedure gave a number of peaks in the HPLC trace. However, when the two procedures were combined, the unrelated peaks were successfully eliminated indicating that different "impurities" were eliminated by the two clean-up procedures.

In contrast to earlier HPLC for 5-S-cysteinyldopa determination [10, 11] we included Na_2EDTA in the mobile phase. This markedly improved the unsteady baseline and lowered the baseline current from about 5—20 nA to less than 1 nA. We chose an EDTA concentration of 0.1 mmol/l in accordance with Moyer and Jiang [18]. In a recent paper Warsh et al. [19] stated that this is a rather high concentration, which causes the detector responses to deteriorate very rapidly. However, we have not noted such an effect with our mobile phase.

With the clean-up we describe here we had the goal to obtain a clean 5-S-cysteinyldopa eluate, and therefore other catechols were not recovered. With the procedure finally adopted the use of α-methyldopa or isoproterenol as internal standard [10] was therefore precluded. We conducted experiments to add these catechols to the final eluates for evaluation of the chromatograms.

We found however (data not shown) that evaluation of the chromatograms by peak height ratio did not improve the precision to any appreciable extent in comparison with peak height evaluation of 5-S-cysteinyldopa (C.V. = 1.04%). This was in accordance with the findings reported by Haefelfinger [20] who showed that in HPLC with automatic injection with complete loop filling (100 μl) the precision of the injection was not the limiting factor for the reproducibility and the internal standard brought no evident advantages.

For the quantitative determination of urinary 5-S-cysteinyldopa we had to determine the individual recovery. We solved this problem by adding tritium-labelled 5-S-cysteinyldopa to the sample and counting the radioactivity before and after the clean-up. This extra step may be considered as a drawback, especially since tritium-labelled 5-S-cysteinyldopa first had to be synthesized. However, we found that tritiated 5-S-cysteinyldopa could easily be synthesized for this purpose and it was stable for several months. The availability of such preparations was also very useful for the development of this method, and may be of importance in other, for example in vivo, studies.

In Table II the results we obtained for urinary excretion of 5-S-cysteinyldopa in healthy subjects can be compared with earlier studies [21—23] using the fluorimetric method [8]. We could not demonstrate any significant difference between men and women as in the earlier studies. This may depend on the rather limited number studied. The absolute amounts excreted, however, were in quite good accordance with earlier findings. This indicates that from the standpoint of evaluating patients' results, similar reference values may be used in clinical practice.

TABLE II

MEAN URINARY EXCRETION OF 5-S-CYSTEINYLDOPA IN HEALTHY SUBJECTS

Number of subjects in parentheses.

Reference	μmol per 24 h		
	Men	Women	Men and women
Agrup et al. [21]	0.32 (30)	0.25 (46)	0.27 (76)
Morishima and Hanawa [22]	0.36 (10)	0.25 (10)	0.30 (20)
Graef and Paul [23]	—	—	0.28 (45)
Present study	0.36 (12)	0.33 (12)	0.34 (24)

ACKNOWLEDGEMENT

This study was supported by grants from The Medical Society of Linköping.

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