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AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF 5-S-CYSTEINYL-3,4-DIHYDROXYPHENYLALANINE IN URINE

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SUMMARY

An automated high-performance liquid chromatographic (HPLC) method has been developed for measurement of 5-S-cysteinyl-DOPA in urine (DOPA = 3,4-dihydroxyphenylalanine). The urinary sample was injected into an HPLC boronate column. With a mobile phase of 0.1 *M* phosphate buffer containing 0.2 m*M* disodium ethylenediaminetetraacetate (Na_2EDTA) (pH 6.0) mixed with methanol (9:1), 5-S-cysteinyl-DOPA was adsorbed while most other compounds were washed away. By column switching, the column flow was reversed and 5-S-cysteinyl-DOPA was desorbed by a mobile phase of 0.1 *M* formic acid and 0.2 m*M* Na_2EDTA at pH 3.0 and chromatographed on a reversed-phase column. The precision, as estimated from repeated analysis of an urinary sample and from duplicate analysis of a number of samples, ranged from 1.4 to 5.2% (coefficient of variation), and the analytical recovery was 93 ± 4.1%. The method is suitable for use in the clinical laboratory.

INTRODUCTION

Pigment formation in melanocytes proceeds by a number of reactions and the first part of the metabolic pathway is fairly well known[1,2]. The enzyme tyrosinase first hydroxylates tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and then oxidizes the latter compound to dopaquinone[3]. This compound is an highly reactive electrophilic intermediate which can undergo an internal ring closure to give indole derivatives[4]. In the presence of thiols the SH group reacts by a nucleophilic attack on dopaquinone to give DOPA-thioether compounds. Several such compounds are known, and among them 5-S-cysteinyl-DOPA is quantitatively dominant[5]. The urinary excretion of this compound increases during UV stimulation[6,7], and its measurement is of great value for the study of melanoma metastases in patients with melanoma[8].

A number of high-performance liquid chromatographic (HPLC) methods for the determination of 5-S-cysteinyl-DOPA has already been developed[9–14]. Recently, we described the determination of 5-S-cysteinyl-DOPA in human urine by direct

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injection in a coupled-column HPLC system with electrochemical detection (ED)[15]. In this method, the crude urinary sample was manually injected directly into a purification column of silica to which 3-aminophenylboronic acid is covalently bonded. After on-column washing with a mobile phase of 0.1 *M* phosphate buffer and 0.2 m*M* Na_2EDTA (pH 6.0) mixed with methanol (9:1), the eluent was changed to a mobile phase of 0.1 *M* formic acid and Na_2EDTA (pH 3.0) which desorbed 5-S-cysteinyl-DOPA and transferred it to an analytical reversed-phase column. We have now investigated the prerequisites for automated analysis of 5-S-cysteinyl-DOPA in urine and report the validation of that method.

EXPERIMENTAL

Materials

The catecholamines, dopamine (3-hydroxytyramine hydrochloride), norepinephrine (L-norepinephrine bitartrate), epinephrine (L-epinephrine bitartrate) and 3,4-dihydroxybenzylamine (DHBA) hydrobromide, as well as 3,4-dihydroxyphenylacetic acid (DOPAC) were obtained from Sigma (St. Louis, MO, U.S.A.). 3,4-Dihydroxy-L-phenylalanine (L-DOPA) was from Merck (Darmstadt, F.R.G.). The DOPA-thioether compounds, 5-S-, and 2-S-L-cysteinyl-L-DOPA and 2,5-S,S-di-L-cysteinyl-L-DOPA[16], 5-S-D-cysteinyl-L-DOPA[17], 5-S-L-cysteinylglycine-L-DOPA[18] and 5-S-glutathionyl-L-DOPA[18] were synthesized as described earlier.

Sodium dihydrogenphosphate, disodium hydrogenphosphate, disodium ethylenediaminetetraacetate (Na_2EDTA), sodium metabisulphite and hydrochloric acid were from Merck, methanol from Rathburn Chemicals (Walkerburn, U.K.) and formic acid from Fluka (Buchs, Switzerland).

Stock solutions (1 m*M*) of L-DOPA, epinephrine, norepinephrine, dopamine and dihydroxybenzylamine were prepared in a solution of 10 m*M* hydrochloric acid and 0.2 m*M* Na_2EDTA. Stock solutions of DOPA-thioethers were prepared in 1 *M* hydrochloric acid solutions. Working solutions were prepared by dilutions in water, except for the 5-S-L-cysteinyl-L-DOPA standard, which was prepared in 0.1 *M* acetate buffer, containing 0.2 m*M* Na_2EDTA (pH 4.0).

Mobile phases

I, pH 6.0. A 0.1 *M* phosphate buffer, containing 0.2 m*M* Na_2EDTA, was prepared from sodium dihydrogenphosphate and disodium hydrogenphosphate and adjusted to pH 6.0. This buffer was mixed with methanol (9:1) and suction filtered through a 0.45-μm cellulose acetate filter (Sartorius, Göttingen, F.R.G.). Similar mobile phases with other pH values were prepared for experimental purposes.

II, pH 3.0. A solution of 0.1 *M* formic acid and 0.2 m*M* Na_2EDTA was adjusted to pH 3.0 with sodium hydroxide. The solution was filtered as for mobile phase I.

The components of the chromatographic system were all from Waters Assoc. (Milford, MA, U.S.A.). The boronate affinity column (35 mm × 3.1 mm I.D.) was packed with acetylated boronic acid-silica (ABA-silica) from Perstorp Biolytica (Lund, Sweden). This material consists of LiChrosorb (10 μm) to which 3-aminophenylboronic acid has been bound covalently[19]. The analytical column (250 mm × 4.6 mm I.D.) was a Supelcosil C_{18} (5 μm) column from Supelco (Bellefonte, PA, U.S.A.). The system configuration is depicted in Fig. 1. It consisted of a Model 590

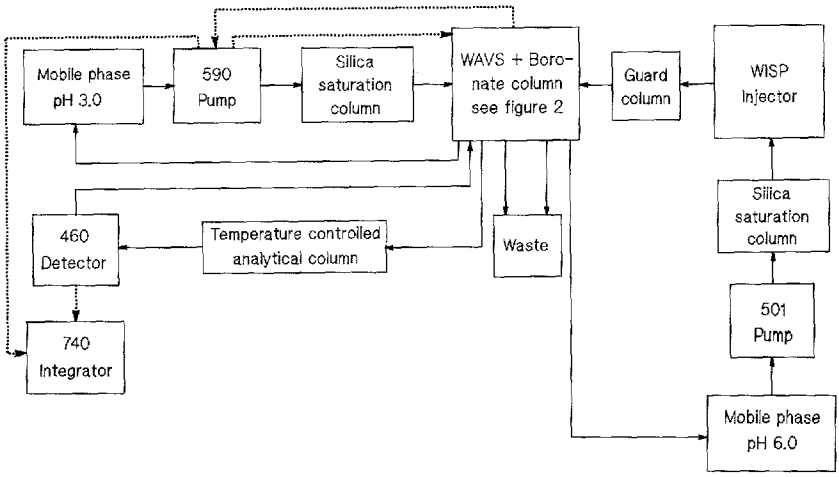


Fig. 1. System configuration for automated determination of 5-S-cysteinyl-DOPA. Dotted lines indicate electrical connections, and solid lines indicate fluid connections. All components were from Waters Assoc.

solvent delivery system, pumping the mobile phase II to the analytical column. This pump was also programmed to control the operation of the WAVS valve station. The samples were transported from the WISP 712 autoinjector to the boronate column with a Model 501 HPLC pump. The silica saturation columns (150 mm × 4.6 mm) before the WAVS and WISP were dry-packed with Polygosil C_{18}, 40–63 μm (Macherey-Nagel, Düren, F.R.G.), and the guard column before the boronate column was a 15 mm × 3.2 mm New Guard RP-18 Column from Brownlee Labs. (Santa Clara, CA, U.S.A.). The analytical column was maintained at 28°C by a Temperature Controle Module thermostat and column heater.

Detection was performed with a glassy carbon working electrode at +0.60 V *versus* Ag/AgCl/3 *M* KCl using a Model 460 electrochemical detector (Waters Assoc.). Assuming a two-electron oxidation of cysteinyl-DOPA, we obtained *ca.* 6% conversion in the electrochemical cell, calculated as A/nFN, where A = area of the chromatographic peak expressed in coulombs, n = number of electrons involved in the electrochemical process (here $n = 2$), F = Faraday's constant, 96 500 C/mol, and N = amount of cysteinyl-DOPA injected in moles.

Integration and calculations were performed by a Model 740 data module from Waters Assoc.

The connections of the WAVS valve station are shown in Fig. 2, upper panel, and an automated sample cycle is described in the same figure, lower panel.

Manual injection was also performed by using the high-pressure valve 1 as an injection valve. This configuration was used to study the recovery from the boronate column.

Methods

Urine was collected for 24 h in bottles containing 5 ml of 0.7 *M* thymol in 2-propanol[20] to inhibit bacterial growth. Aliquots of the urines were stored at −20°C until analyzed.

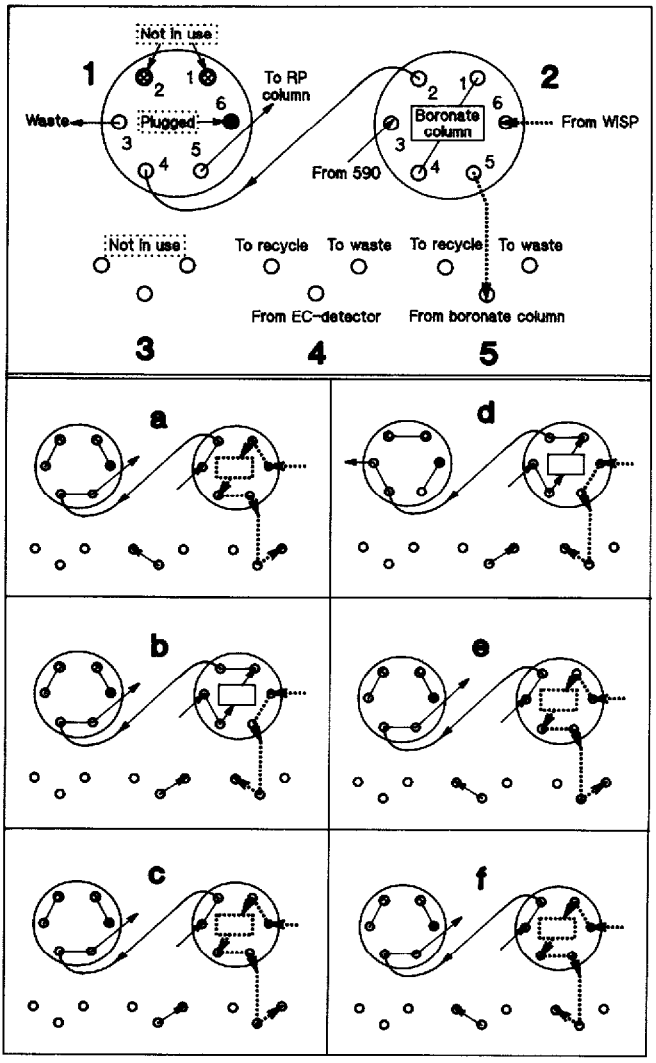


Fig. 2. Usage of the Waters automated valve station. We used both the high-pressure valves (1 and 2) and two of the three low-pressure valves (4 and 5). Upper panel: plumbing for automated analysis of 5-S-cysteinyl-DOPA. This is a key to the lower panel. Lower panel: operation cycle of the automated determination of 5-S-cysteinyl-DOPA. The two pumps are pumping at a flow-rate of 1.0 ml/min unless stated otherwise. Solid lines indicate mobile phase II and dotted lines indicate mobile phase I. (a) Sample is introduced into and adsorbed onto the boronate column. Interfering compounds are washed away for 8 min. (b) The flow direction of the boronate column is reversed, and mobile phase II desorbs cysteinyl-DOPA for 0.7 min. (c) Desorption is completed, and cysteinyl-DOPA is chromatographed on the analytical column. The 501 pump is not turned off. (d) Cysteinyl-DOPA has now been detected. The 590 pump is programmed to decrease the flow-rate slowly to 0. The high-pressure valve 1 is switched so that the boronate column can be washed for *ca.* 3 min at 2 ml/min with mobile phase II. The total wash volume is 6.9 ml. (e) Reequilibration for 10 min of the boronate column is started. (f) The system is now ready for a new injection. Both mobile phases are recycled until the sample is injected.

Urine samples were thawed in cold water and mixed thoroughly, and a 10-ml aliquot was clarified by centrifugation at 1300 *g* for 5 min. A measured volume of the urine was then diluted in two volumes of a 100 m*M* acetate buffer, containing 0.2 m*M* Na_2EDTA (pH 4.0). The diluted urine was transferred to ampoules and placed in the autoinjector.

In routine analysis we usually started with a standard sample, followed by urines, and a new standard sample for each ten samples. If the results were above 3 μM and quantitative analysis was needed, the sample was further diluted in the acetate buffer (see above) and reanalyzed.

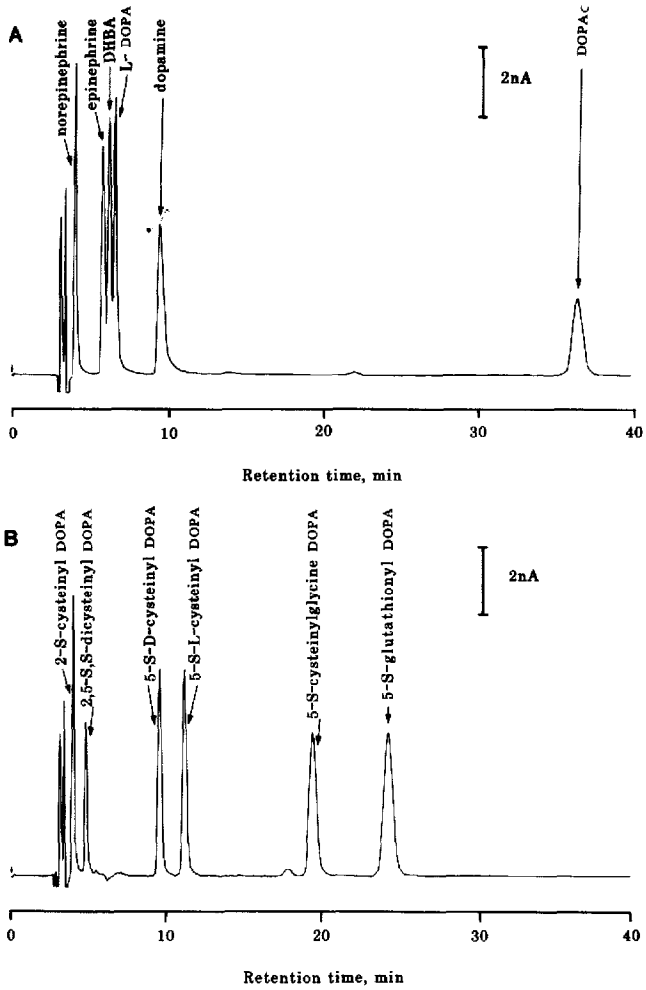


Fig. 3. Separation of synthetic catecholamines, L-DOPA and DOPA-thioethers. Injection was performed directly into the analytical column. (A) Norepinephrine, epinephrine, DHBA, L-DOPA, dopamine and DOPAC. (B) 2-S-Cysteinyl-DOPA, 2,5-S,S-dicysteinyl-DOPA, 5-S-D-cysteinyl-L-DOPA, 5-S-L-cysteinyl-L-DOPA, 5-S-cysteinylglycine-DOPA and 5-S-glutathionyl-DOPA.

RESULTS

Fig. 3 depicts the separation of catecholamines, L-DOPA and DOPA-thioethers on the analytical reversed-phase column only. There was satisfactory separation of the compounds of interest, except that dopamine and 5-S-D-cysteinyl-L-DOPA were not separated, both having a retention time of *ca.* 9.6 min.

Fig. 4 depicts the variation of the peak area for 5-S-L-cysteinyl-L-DOPA as a function of the desorption time. Complete elution was achieved after 0.7 min. Since a ghost peak was also found which increased with time, we chose 0.7 min as our standard desorption time.

When injection of the compounds into the boronate column was followed by column switching, satisfactory separation was also obtained (Fig. 5). In general, the retention times changed very little, and no broadening of peaks occurred. Thus for 5-S-L-cysteinyl-L-DOPA, injected directly into the analytical column or by column switching, the efficiency, $N = 16\ (t_R/w_b)^2$, was about 5500. The resolution, $R_S = 2\Delta t/(w_{b1} + w_{b2})$ (w_b is the peak width at its base, Δt is the difference in retention time of the two peaks), for the separation of 5-S-L-cysteinyl-L-DOPA and 5-S-D-cysteinyl-L-DOPA, as tested for the two types of injection, was about 2.9, and the asymmetry factor, B/A at 10% peak height[21], varied between 1.1 and 1.2 with both types of injection, even after 355 injections. No deterioration of the column was observed after 1000 injections.

From a comparison of Fig. 5 with Fig. 3 it is seen that L-DOPA and DOPAC were not detected after adsorption on the boronate gel at pH 6.0, and a ghost peak was obtained with a retention time of 7.4 min when the boronate column was eluted into the reversed-phase column.

Prolonged washing of the boronate column at pH 6.0 resulted in almost complete elution of the catecholamines, while 5-S-cysteinyl-DOPA remained on the column (Fig. 6).

We compared the peak area of 5-S-L-cysteinyl-L-DOPA after injection with a calibrated loop directly into the analytical column and via the boronate column. The recovery from the boronate column was 92%.

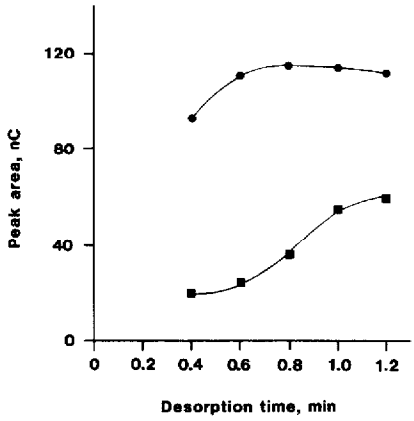
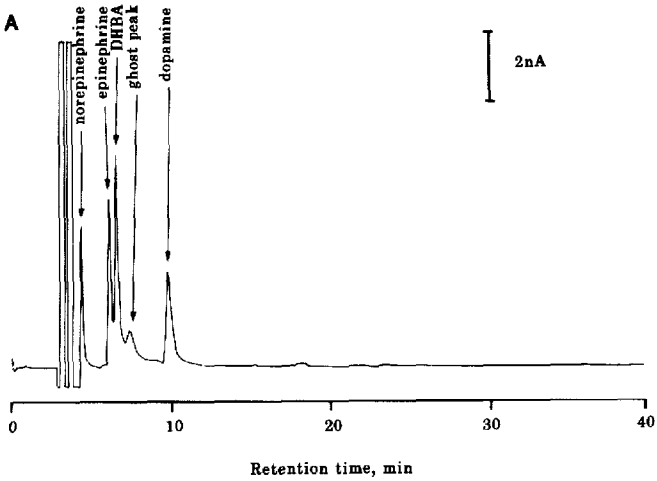


Fig. 4. Desorption of 5-S-cysteinyl-DOPA from the boronate column as a function of the elution with mobile phase II. (●) 5-S-L-cysteinyl-L-DOPA; (■) ghost peak.



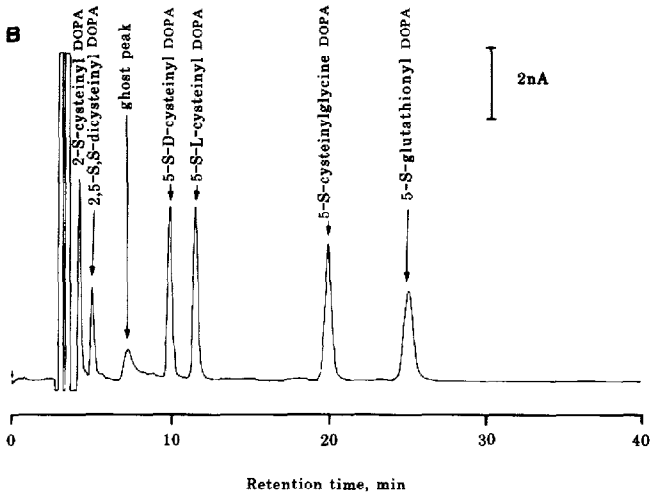


Fig. 5. Separation of compounds of interest after injection into the boronate column and chromatographed after column switching on the reversed-phase column. (A) and (B) as in Fig. 3.

The carry-over was estimated by measurement of the 5-S-L-cysteinyl-L-DOPA peak, obtained in a chromatogram after injection of pure water, following chromatography of a standard of 5-S-cysteinyl-DOPA at a concentration of 50 μM. The carry-over was 0.87% in the first water sample, and 0.22% in the second water injection. Trace amounts of 5-S-cysteinyl-DOPA were also detected in the following two chromatograms corresponding to pure water injection. Because the 5-S-cysteinyl-DOPA concentration can vary considerably in melanoma urines, we did not accept this carry-over, but washed the boronate column with mobile phase II after each injection. By this procedure the carry-over was reduced to 0.03%.

In our first application of a boronate HPLC column to the determination of

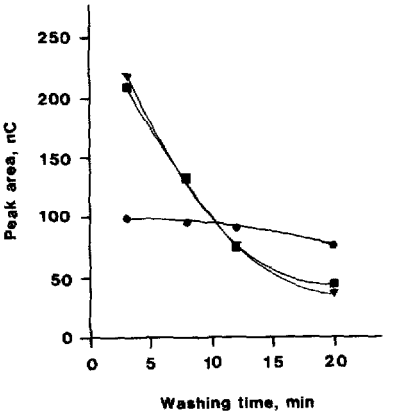


Fig. 6. Stability of 5-S-L-cysteinyl-L-DOPA (●) on the boronate column during prolonged washing, as compared with that of norepinephrine (■) and dopamine (▼). Washing was with mobile phase I, pH 6.0.

5-S-cysteinyl-DOPA we prepared the standard in mobile phase I. The short-term (< 4 h) stability seemed satisfactory, but in extended series 5-S-cysteinyl-DOPA deteriorated, and this solution could not be used for preparing a standard solution because of the risk of oxidation. 5-S-Cysteinyl-DOPA was stable for at least 30 h in 0.1 *M* acetate buffer containing 0.2 m*M* Na_2EDTA (pH 4.0). This was also the case when EDTA was not added. Addition of 5 m*M* sodium metabisulphite to the acetate buffer with EDTA changed the stability of 5-S-cysteinyl-DOPA for the worse. Thus, in subsequent experiments the standard was prepared in 0.1 *M* acetate buffer (pH 4), containing 0.2 m*M* Na_2EDTA.

The choice of optimum washing time depended on the results from the chromatography of urines. We found that a washing time of 8 min was adequate for minimizing the appearance of extraneous peaks. Figs. 7 and 8 depict chromatograms of

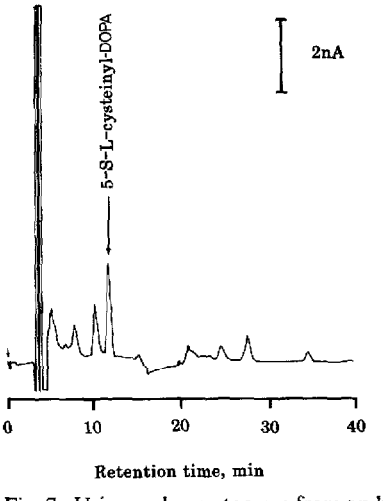


Fig. 7. Urinary chromatogram from an healthy subject. The 5-S-cysteinyl-DOPA concentration was 0.89 *μM*.

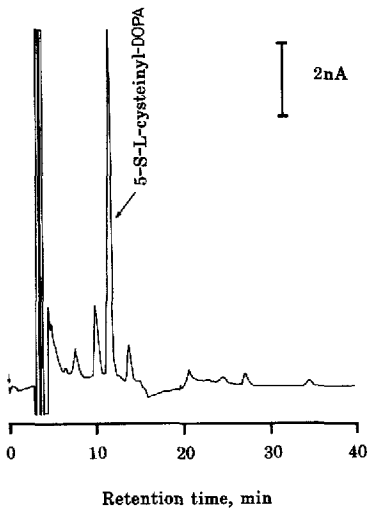


Fig. 8. Urinary chromatogram from a patient with melanoma metastases. The 5-S-cysteinyl-DOPA concentration was 3.42 μM.

urines from an healthy subject and a patient with known melanoma metastases.

The identity of the peak from urine was first confirmed by adding synthetic 5-S-cysteinyl-DOPA to urine and recording of its identical retention. Furthermore, we incubated the urine with the enzyme tyrosinase which oxidizes 5-S-cysteinyl-DOPA to its quinone. This procedure completely abolished the peak and confirmed its authenticity.

We studied the stability of 5-S-cysteinyl-DOPA in urinary samples when applied to the automated sampler standing by for injection. For these studies, we used freshly collected urines, and to obtain fresh alkaline samples, volunteers were given 4–8 g of sodium bicarbonate by mouth 4–8 h before providing urine. It appeared that 5-S-cysteinyl-DOPA in urines at pH $>$ 6.5 was unstable. For routine analysis, we therefore always diluted the urines in two volumes of acetate buffer, which gave a pH value between 4 and 5. This ensured stability of the samples for more than 24 h.

The precision was estimated from repeated (n = 20) analysis of an urinary sample, with standardization before and after ten injections. The mean concentration obtained was 0.767 μM and the standard deviation was 0.011 μM, which gives a coefficient of variation of 1.4%. Furthermore, duplicate analysis of urinary samples with different 5-S-cysteinyl-DOPA concentrations gave similar precision values (Table I). From these data the detection limit for 5-S-cysteinyl-DOPA in urine was calculated as 0.03 μM.

For estimation of the analytical recovery, 5-S-cysteinyl-DOPA in six normal human urines was determined in duplicates (initial concentration 0.18–0.63 μM) and 5-S-cysteinyl-DOPA was added to increase the concentration by 0.59 μM. The increase was estimated to be 0.51-0.57 μM corresponding to a mean ($\pm$ S.D.) recovery of 93.5 ($\pm$ 4.1)%.

Comparison of our results with those obtained by the earlier method[12] showed that the present method gave similar values. Regression analysis gave a straight line

TABLE I

PRECISION CALCULATED FROM DUPLICATE ANALYSIS OF 5-S-CYSTEINYL-L-DOPA IN URINE

Number of samples	*Concentration (μM)*		*Standard deviation (μM)*	*Coefficient of variation (%)*
	Mean	*Range*		
32	0.30	0.08–0.52	0.016	5.2
22	0.97	0.55–3.1	0.013	1.4
5	27	5.4–98	0.39	1.4

according to the equation:

$$\text{present method} = 0.96\ (\text{comparison method}) - 0.05\ \mu M\ (n = 58,\ r = 0.97)$$

About 25 determinations can be performed per day with only a few hours of technicians' time. This is a major advantage compared to earlier non-automated methods.

DISCUSSION

Chromatographic separation of catecholamines and catecholic amino acids by use of boric derivatives bound to a support matrix was described more than 10 years ago[22,23]. A boronic affinity HPLC column was later developed[19], and we recently reported its use in a manual column-switching method for determination of 5-S-L-cysteinyl-L-DOPA. Thus, without prior purification, the urine sample is injected into the system at pH 6.0, and 5-S-L-cysteinyl-L-DOPA is adsorbed on the boronate column. By switching to another mobile phase (pH 3.0), the 5-S-L-cysteinyl-L-DOPA is desorbed and chromatographed on the reversed-phase column. Such methods have also been published for the determination of, *e.g.*, dopamine, norepinephrine, epinephrine and DOPAC[24–27].

De Jong *et al.*[27] have described the prerequisites for on-line sample pretreatment on small alumina and dihydroxyboryl-silica for the analysis of catecholamines. The retention of catecholamines and derivatives was determined by injection of standards, applying eluents of pH 2–8. At low pH (< 5), neither the catecholamines nor their derivatives were retained. At pH 8, only derivatives possessing the catechol function (DOPA, α-methyl-DOPA, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethylene glycol, 3,4-dihydroxyphenylethanol), and compounds containing the amine function (normetanephrine, metanephrine and 3-methoxytyramine) and the classical catecholamines (dopamine, norepinephrine and epinephrine) were retained. Only catecholamines were retained very strongly. The authors concluded that for preconcentration a pH of *ca.* 8 is the best choice.

Cysteinyl-DOPA contains, in addition to the catecholic function, two amino acid groups. This may be the reason why its adsorption on the boronate affinity column is even stronger than that of norepinephrine[15]. We therefore chose a lower pH value (pH 6.0) for adsorption. At this pH value 5-S-cysteinyl-DOPA is retained, while norepinephrine and dopamine are partly eluted (Fig. 6).

We have also tried to use a smaller boronate affinity column (36 mm × 2.0 mm

I.D.). Also with this column satisfactory results were obtained. The precision is similar, but the analysis time cannot be shortened, because the flow-rate has to be reduced during elution at increased pressure.

As is seen from Fig. 3, the retention of dopamine on the analytical column was nearly identical to that of 5-S-D-cysteinyl-L-DOPA. In earlier methods[12,14] we employed this 5-S-cysteinyl-DOPA diastereomer as an internal standard, but due to interference from dopamine it would be unsuitable in the present method. Although use of an internal standard is very convenient in some situations, its use in the present method is unnecessary.

Melanin production has classically been considered to follow two pathways, the DOPA-thioether pathway, which gives pheomelanin, and the indolic pathway, which gives eumelanines. The analysis of melanocyte metabolites as markers of malignant melanoma has been performed since the days of Thormählen, *ca.* 100 years ago[28]. The Thormählen test is positive for indole compounds with a special substitution pattern[29]. Thus this test is not specific for a single compound but for a group of substances, and in the last decade it has been replaced by analysis of 5-S-cysteinyl-DOPA for monitoring melanoma patients[2].

In recent years, modern methods for the analysis of individual indole compounds have been published[4,30]. An increase in their concentrations was found for several of the eumelanin metabolites in the urines of patients with known melanoma metastases[30], but low values have also been reported for a patient with extensive metastases and an high urinary 5-S-cysteinyl-DOPA concentration[31]. Thus, at this time, 5-S-cysteinyl-DOPA seems to be the best marker of malignant melanoma metastasis.

The rapid increase in the incidence of malignant melanoma reported by several countries will greatly increase the demand for a clinical routine method in the near future. The automated method presented in this paper is well suited for the follow-up of patients with suspected melanoma metastases.

ACKNOWLEDGEMENTS

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