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Determination of 5-*S*-cysteinyldopa in plasma and urine using a fully automated solid-phase extraction–high-performance liquid chromatographic method for an improvement of specificity and sensitivity of this prognostic marker of malignant melanoma

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Abstract

5-*S*-Cysteinyldopa (5-SCD) in plasma and urine was determined by means of a newly developed method. This method incorporates optimized conditions for blood collection and storage, as well as a new extraction and separation technique, required for the strong oxidation and light sensitive 5-SCD. The new aspects of the method are the following: immediate centrifugation and freezing of the samples after blood collection, fully automatical solid-phase extraction (SPE) with phenylboronic acid (PBA) cartridges and immediate HPLC injection of the eluate, nearly complete exclusion of light and air–oxygen during extraction, constant sample cooling, use of the more suitable internal standard 5-*S*-D-cysteinyldopa and easy, sensitive and selective HPLC conditions (RP18-column with isocratic separation and electrochemical detection). The method has a linear range from 0.25 to 50 $\mu g\,l^{-1}$ and 25 to 5000 $\mu g\,l^{-1}$ for plasma and urine samples, respectively, a limit of detection of 0.17 $\mu g\,l^{-1}$, intra-assay variabilities from 1.7 to 3.6%, inter-assay variabilities from 4.0 to 18.3% and an average relative recovery of 103.5% for plasma and 105.4% for urine samples. In our study the measured 5-SCD concentrations of patients with melanomas at various stages correlated better with their clinical pictures than described in literature up to date. The results were obtained in comparison to patients with other skin tumors and in comparison to healthy control persons.

Keywords: Plasma; Urine; Metastatic melanoma; 5-*S*-cysteinyldopa

1. Introduction

The incidence and the mortality rates of malignant melanoma are strongly increasing world-wide, especially in the countries of the southern hemisphere. In Germany, the incidence is 15 of 100 000 people and it is expected to double in the next 10–15 years [1].

Tyrosinase, a specific enzyme of melanocytes and melanoma cells, catalyzes the oxidation of tyrosine to dihydroxyphenylalanine (DOPA) and dopachinone, which in the presence of cysteine yields 5-S-L-cysteinyl-L-dopa (5-SCD). The large amount of 5-

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SCD is further oxidized to pheomelanin pigments. Minor amounts of 5-SCD pass over to the blood stream and are excreted mainly in the urine. With the estimation of 5-SCD in plasma and urine, information can thus be obtained about the activity of tyrosinase in melanoma cells and about the progression of melanoma [2–4].

The first report about an increased excretion of 5-SCD in urine of patients with metastatic melanoma was published in 1973 [5]. In the study, it was clearly demonstrated that a positive correlation exists between 5-SCD concentration in urine and the progress of the melanoma [6–13]. For instance, six of 43 patients (14%) in stage III (locoregional metastases) and 45 of 68 patients (62%) in stage IV (advanced metastatic disease) showed a pathological 5-SCD excretion (>400 $\mu g\ day^{-1}$) [9].

With the analysis of plasma or serum samples [14–17], a progress in the sensitivity of this marker was reached. This is the case because 5-SCD in plasma reflects the metastases of melanoma at an earlier point than in urine [12,18] and furthermore reveals them earlier than the clinical picture of a patient and earlier then other laboratory tests like scintigraphy or echography [11]. As opposed to this, Hara et al. [19] observed plasma 5-SCD concentrations that were not correlated to melanoma stages and that were in a pathological range only for patients in the stages II and IV. Contradictory results were obtained also for normal values in plasma [12].

Several analytical methods exist to estimate 5-SCD in plasma and urine [5,14–18,20–23]. From 1978 to the present, HPLC-separation with electrochemical detection has become the exclusive technique for 5-SCD determination. Mostly alumina extraction was applied prior to HPLC-analysis [14,16–18,20,21,23], sometimes coupled with an additional liquid–liquid-extraction step. But also enrichment methods with phenylboronate affinity gel have been described [15,22]. However, all methods had one or more disadvantages: either the extraction and/or separation methods were tedious [14–16,23], or large plasma sample volumes were required [14,22], or relatively unsuitable I.S. were used (isoproterenol, αMD) [14,16,20] or an oxidation and light protection during extraction was not possible (in case of all alumina extractions).

In spite of the impressive results for sensitivity and also for specificity, well known for a long time, this marker was not successful in clinical praxis. The key event for us was the analysis of samples from a preliminary study testing the suitability of a few biochemical parameters as marker for the malignant melanoma. From these 134 serum samples (collected under 'normal' clinical conditions, i.e. without temperature-, oxidation- and light-protection and thawed and frozen several times) of melanoma patients in the stages III and IV, only 11 (8%) showed a measurable 5-SCD signal at all, mostly near the limit of detection. Only one sample was found with a slight pathological 5-SCD value.

Because of its chemical structure, 5-SCD is extremely sensitive to oxidation and light exposure. From this point of view, the above results were not unexpected. It is amazing, that these properties of 5-SCD were given little or no attention in prior literature, what concerns the pre-analytical and the analytical phase. It can be supposed that many of the problems and contradictions of sensitivity and specificity of 5-SCD as marker for the malignant melanoma, especially in plasma, originate from inappropriate analytical conditions and less from the biochemical suitability. To overcome these problems, we developed a completely new analytical method for determination of 5-SCD in plasma and urine that took into account the entire analytical procedure.

2. Experimental

2.1. Chemicals and supplies

The standard solution of 5-*S*-L-cysteinyl-L-3,4-dihydroxyphenylalanine (5-*S*-L-cysteinyl-L-dopa, 5-SCD) and plasma controls (ClinRep®) were purchased from Recipe (München, Germany). L-3,4-Dihydroxyphenylalanine (L-DOPA), L-cysteine, D-cysteine, 2-methyl-3-(3,4-dihydroxyphenyl)-L-alanine (αMD), tyrosinase and [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid (EGTA) were obtained from Sigma (München, Germany). EDTA-$(NH_4)_2$ we obtained from Fluka (München, Germany). All other used chemicals were purchased from E. Merck (Darmstadt, Germany) and were analytical or HPLC-grade. Water was purified by a Milli-Q-water purification system (Millipore, Esch-

born, Germany). Solid-phase extraction (SPE) columns (Bond Elut®) with phenylboronic acid (PBA), 1 ml, containing 100 mg of sorbent, were purchased from ICT (Bad Homburg, Germany). For complete passage of solutions through the cartridges in SPE, we operated with pressure of nitrogen.

The used 1 m*M*, 10 m*M* and 0.3 *M* HCl and the 10 m*M* Tris buffer solutions contained 2 m*M* EDTA-$(NH_4)_2$. The pH of the used Tris buffer solutions was 8.30. The oxidation protection solution (OPS) consisted of 0.25 *M* EGTA and 0.2 *M* GSH, pH=7.0 adjusted with NaOH.

2.2. Instrumentation

The HPLC system consisted of the following modules: a L-6200 gradient pump, a column thermostat T-6300, a D-6000 interface with the LiChroGraph-HPLC-software (all from Merck/Hitachi, Darmstadt, Germany), a dual channel electrochemical detector BAS LC4C (Axel Semrau, Sprockhövel, Germany) and an on-line degasser DG1310 (VDS Optilab, Montabaur, Germany).

For the solid-phase extraction procedure and the automatic on-line sampling into the HPLC system, an ASPEC XLi with a model 402 syringe pump and with X-tray-software (both from Abimed Analysentechnik, Langenfeld, Germany) was used. The racks were cooled to 4°C with an external device. All liquid transfers were performed through a long stainless steel cannula and a 10 ml coiled tubing with a 10 ml syringe and water as the hydraulic agent. For our purpose, the configuration of the ASPEC was as follows: six different racks, three additional ports for further solutions, a 3-way low-pressure valve for working with nitrogen and a high-pressure HPLC injection valve. The racks were not only cooled, but also protected from light with aluminum foil.

2.3. Standards

The synthesis and purification of 5-*S*-L-cysteinyl-L-dopa and 5-*S*-D-cysteinyl-L-dopa (D-CD) were carried out according to the procedure of Huang et al. [24], however for the purification of the reaction mixture we applied our own SPE-method (see 2.5).

In the case of 5-SCD, we used either the standard solution from Recipe (20 $\mu g\,l^{-1}$) or we diluted our self-prepared 5-SCD stock solution (approximate 200 $mg\,l^{-1}$ in 0.01 *M* HCl). The latter was quantified with aid of the Recipe standard.

The stock solution of our normal internal standard, D-CD, had a concentration of roughly estimated 1000–1500 $mg\,l^{-1}$.

The concentration of the stock solution of the tested second I.S., αMD, was 1 $g\,l^{-1}$ in 0.01 *M* HCl (prepared monthly, storage at 4°C). In working practice, we used a mixture of both internal standards with a suitable working concentration (αMD: 100 $\mu g\,l^{-1}$, in 1 m*M* HCl). The stock solutions of our self-prepared substances were stable for at least six months when stored at −80°C. The working standards were prepared daily from the stock solutions.

2.4. Sample collection and storage

6 ml blood samples of healthy volunteers, melanoma patients or patients with other skin tumors were drawn by venipuncture into vacuum tubes containing EDTA. Plasma was separated by immediate centrifugation (10 min, 1500 *g*, 4°C), transferred to new polyethylene tubes, containing 100 μl 0.2 *M* EDTA-Na_2 and 100 μl 0.5 *M* $Na_2S_2O_5$ and stored at −80°C.

For the collection of 24 h-urine samples, we used 2000 ml bottles, containing 10 ml 6 *M* HCl and 1g $Na_2S_2O_5$. During the collection, these bottles were kept cool and stored in the dark. After finishing the collection, a 10 ml aliquot of the urine was transferred to a polyethylene tube and stored at −80°C.

2.5. Sample preparation

The PBA-SPE-cartridges for aqueous standards and urine samples can be reused four times without change in recoveries of interested substances; those for plasma samples can only be used once. All preparation steps were performed with polyethylene or polypropylene materials, except for the use of the stainless steel cannula of the ASPEC.

The SPE was basically made as follows: firstly, each PBA-SPE-cartridge was washed with 1 ml heptane and 1 ml acetone to remove possible substances remaining from the manufacturing process. The normal sorbent conditioning consisted of each 1

ml methanol, 1 ml 0.3 *M* HCl and 1 ml methanol again, followed by 2 ml 10 m*M* Tris buffer. For sample preparation, we used 1000 μl plasma (or 1:100 diluted urine or 1 m*M* HCl for standard samples), mixed with 200 μl 1 m*M* HCl (or appropriate standard working solution for standard samples), 20 μl OPS, 50 μl suitable solution of the mixed I.S. and 330 μl 1 *M* Tris buffer. After loading the cartridges with these mixtures, a brief application of pressure (nitrogen) was used to complete the passage of solutions through the cartridges. The SPE-columns were each washed with 4 ml 10 m*M* Tris buffer, followed by an application of pressure (nitrogen). The elution of the interested substances was done with each 1 ml 0.3 *M* HCl, followed once again by an application of pressure (nitrogen). For cartridges of standards or urine analysis, a regeneration program can be applied (add to each 2 ml 0.3 *M* HCl and 2 ml methanol).

Automated SPE was performed as follows: first, the working solution of the mixed I.S. was prepared in a two-step-procedure. Second, the sample preparation of all samples and standards were performed by mixing the original samples, the mixed I.S. and the OPS. From this point on, the further extraction of the samples was made one by one. The SPE for one sample, after the initial sample preparation had been completed, required 23 min. The configuration of the used ASPEC is shown in Fig. 1.

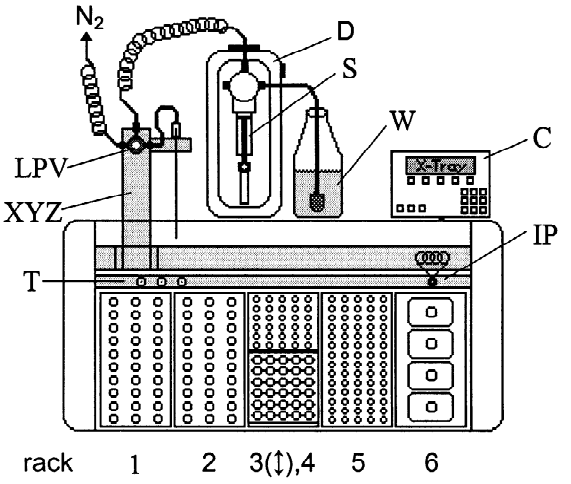

Fig. 1. Configuration of the ASPEC XLi, used for SPE and HPLC-injection. D: syringe pump dilutor 402; S: 10 ml syringe; W: bottle with water as hydraulic agent; C: keypad controller with X-Tray software; IP: HPLC-injection port with a Rheodyne 7010 high-pressure valve (500 μl sample loop); T: transfer ports for solutions; XYZ: vertical arm for motion in xyz-direction with stainless steel cannula; LPV: low-pressure valve for application of nitrogen (N_2).

2.6. Chromatographic conditions

For the HPLC separation, we used a 250×4 mm, 5 μm, LiChrospher 100 RP18 endcapped LiChroCAR-T-column (Merck), protected with a guard column cartridge 5×4 mm (Knauer, Berlin, Germany), filled with the same material. For on-line purification of the eluents, we used a precolumn 30×4 mm (Knauer) between pump and ASPEC-autosampler, that we filled ourselves with LiChrospher 100 RP18, 10 μm particle size.

The chromatographic separation was performed under isocratic conditions with eluent A. After 11 min, when the latest peak of interest (αMD) had left the analytical column, the pump switched to 100% of the stronger eluent B, to purge all late peaks of interference from the column. After 20 min, the pump switched back to eluent A. The total chromatographic run time was 30 min.

The compositions of the eluents were for eluent A: 0.15 *M* KH_2PO_4, 100 mg l^{-1} EDTA-$(NH_4)_2$, 5% MeOH (v/v), pH=2.80 (plasma) or pH=2.65 (urine) and for eluent B: 0.15 *M* KH_2PO_4, 100 mg l^{-1} EDTA-$(NH_4)_2$, 20% MeOH (v/v), pH=3.00. An exact pH-adjustment for the eluents A was necessary to achieve a good separation of the interesting from interfering peaks. The flow-rate of both eluents was 1 ml min^{-1}. The temperature of the column thermostat was set to 35°C. The injection volume (with a 500 μl sample loop) was 400 μl. For detection, we used a dual channel (parallel arrangement) amperometric glassy carbon electrode. The reference electrode was a Ag/AgCl (3 *M* NaCl) model. The potentials for channel 1/channel two were 500/450 mV (range 2 nA). The electronic filter was set to 0.06 Hz.

2.7. Data analysis and quantification

The quantification was performed on the basis of the 5-SCD standard solution from Recipe (20

$\mu g\,l^{-1}$). 5-SCD concentrations were calculated relative to the peak areas of the I.S. (D-CD or αMD), in order to correct 5-SCD loss during sample preparation. To estimate sample concentrations we used for daily routine analysis a three-point standard calibration curve (based on linear least-square regression, $y = bx + a$) with aqueous standard solutions (0.5, 3.0 and 8.0 $\mu g\,l^{-1}$) including solid-phase extraction.

The correctness of the complete analytical procedure for plasma samples was examined with commercial plasma controls at two levels (5.3 and 12.9 $\mu g\,l^{-1}$) and with a standard addition procedure ($+1.0$ $\mu g\,l^{-1}$) to a blank-plasma. Controls were not available for urine samples, so we performed only a standard addition technique ($+40$ $\mu g\,l^{-1}$ and $+400$ $\mu g\,l^{-1}$) to verify the analysis.

The compounds of interest were identified on the basis of their retention times, the addition of standards and the ratio of peak areas detected at two different potentials.

2.8. Investigations of stability of the 5-SCD concentration in plasma

To test the stability during the extraction process under various conditions of temperature, light and air–oxygen, a commercial available plasma was spiked with appropriate amounts of 5-SCD and aliquoted into 1 ml portions for extraction and analysis in the full charged ASPEC (30 positions). Series with pathological and normal 5-SCD concentrations were measured.

For investigations of the stability of plasma 5-SCD in the eluate after SPE, a plasma sample was spiked with an appropriate amount of 5-SCD, to yield a distinct 5-SCD-peak with an injection volume of only 40 μl, and then normally extracted with the ASPEC. The eluate was injected nine times in a row under conditions of a normal autosampler (room temperature, daylight).

To investigate what influence waiting time before centrifugation has on blood samples, we collected 8 ml blood samples of each 14 melanoma patients in different stages. The blood was drawn by venipuncture into vacuum tubes containing EDTA. In each case, the blood was split in two aliquots into new tubes, containing each 100 μl 0.2 *M* EDTA-Na_2 and 100 μl 0.5 *M* $Na_2S_2O_5$. One aliquot was centrifuged immediately (10 min, 1500 *g*, 4°C), the plasma was transferred to a new polyethylene tube and stored at −80°C. The other one was only centrifuged after a waiting period of 3–4 h at 4°C in the dark, then the plasma was transferred to another new polyethylene tube and stored at −80°C.

3. Results and discussion

Typical chromatograms obtained from the analysis of 5-SCD are shown in Fig. 2. For plasma analysis the retention times for extracted plasma samples and aqueous standards were 7.9 min for 5-SCD (R.S.D. 0.22%), 7.0 min for D-CD (R.S.D. 0.24%) and 10.9 min for αMD (R.S.D. 0.20%). For urine analysis we used another eluent A with slightly different pH value and therefore, we obtained slightly shorter retention times as well for urine samples as for aqueous standards (7.8, 6.9 and 10.7 min; the R.S.D.s were the same as above). The results will be discussed with attention paid to the reference values for normal and pathological concentrations obtained in this work. We consider 5-SCD concentrations <1.6 $\mu g\,l^{-1}$ as a normal range, values between 1.6 and 3.2 $\mu g\,l^{-1}$ as a border range and concentrations >3.2 $\mu g\,l^{-1}$ as a pathological range for plasma samples. The values for 24 h urine excretion are <150 $\mu g\,day^{-1}$, 150–400 $\mu g\,day^{-1}$ and >400 $\mu g\,day^{-1}$.

3.1. Linearity of the detector response and the sample extraction

For the linearity of the pure detector response for aqueous standard solutions, we found the best results with linear fit in the range from 0.1 to 20 $\mu g\,l^{-1}$ (7 data points) both for peak areas, as well as for peak-area ratios related to the I.S. (D-CD). In both cases, the squares of the correlation coefficients *r* were 1.000. At higher 5-SCD concentrations, the peak height was greater than the working range of the detector. We tested the linearity also under analytical conditions (with SPE). In that case, because the recovery of 5-SCD was lower than 100%, we could utilize data points for concentrations to 50

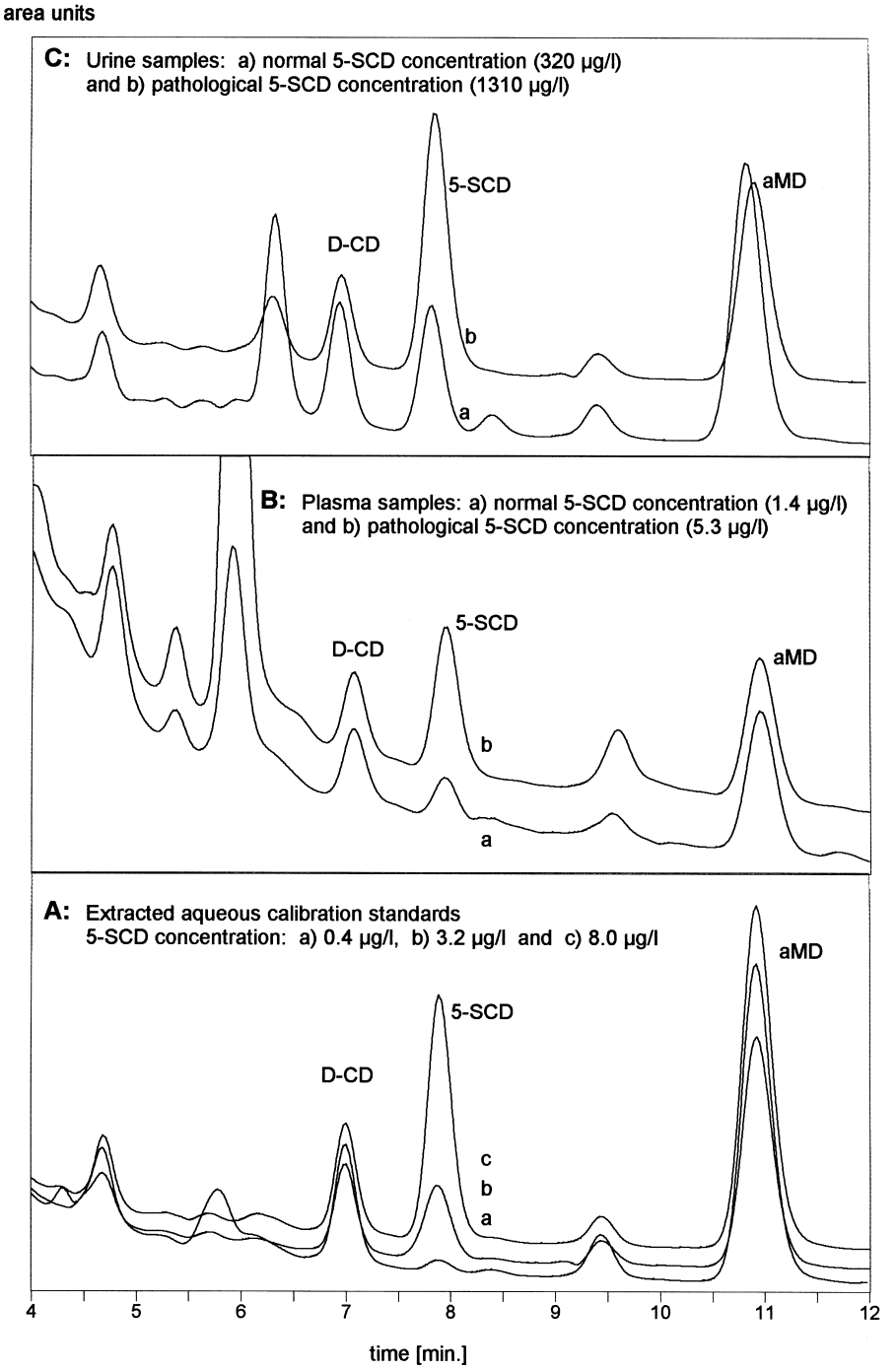

Fig. 2. Typical chromatograms of extracted aqueous standards (A), plasma (B) and urine (C) samples (ECD-channel 1: 500 mV, 2 nA/FS), total runtime: 30 min.

$\mu g\,l^{-1}$. For aqueous standards, spiked blank-plasma and blank-urine samples (0.25, 0.5, 2, 4, 8, 12, 20, 50 $\mu g\,l^{-1}$, for urine concentrations $\times 100$; each concentration with double measurement) the values for r^2 for linear regression using the peak-area ratios and the peak areas, were 0.998 and 1.000, respectively.

3.2. Calibration

We investigated the inter-day reproducibility of the calibration function $y = bx + a$ in the essential range for our application at nine consecutive days. For this purpose the used 5-SCD concentrations for aqueous standards and spiked plasma and urine samples were $+0.4$, $+1.6$, $+4$, $+10$, and $+20$ $\mu g\,l^{-1}$. The corresponding values for the three-point calibration curve for daily routine analysis (5-SCD-concentrations $+0.5$, $+3.0$ and $+8.0$ $\mu g\,l^{-1}$) are given in angular parentheses.

The values are related to the area of the I.S. and are expressed as means$\pm$S.D. The functions were $y = (0.359 \pm 0.026)x - 0.011 \pm 0.023$ [$y = (0.363 \pm 0.031)x - 0.008 \pm 0.028$] for aqueous standard samples, $y = (0.343 \pm 0.017)x - 0.012 \pm 0.020$ [$y = (0.345 \pm 0.020)x - 0.010 \pm 0.022$] for the plasma matrix and $y = (0.386 \pm 0.027)x + 0.073 \pm 0.024$ [$y = (0.383 \pm 0{,}031)x + 0{,}075 \pm 0{,}025$] for the urine matrix. The means$\pm$S.D. of the squares of the correlation coefficients r for these calibrations were for aqueous standard samples 0.9963 ± 0.0021 [0.9969 ± 0.0018], for plasma matrix 0.9924 ± 0.0061 [0.9955 ± 0.0054] and for urine matrix 0.9988 ± 0.0012 [0.9991 ± 0.0005].

As evaluated by Student's t-test ($P = 0.95$), no significant differences exist between the slope in aqueous standards and the slopes in plasma or urine samples. For this reason there was no need for a special matrix calibration.

3.3. Limit of detection (LOD) and limit of quantification (LOQ)

For the calculation of the LOD usually the formulas $y_{LOD} = y_{blank} + 3$ S.D.$_{blank}$ ($P = 0.998$) and then $x_{LOD} = LOD = 3$ S.D.$_{blank}$/slope b were used. In accordance of that is the absolute quantity of a blank value not of importance for the LOD value. For our calculations we used samples with a slight 5-SCD concentration near the expected LOD (aqueous standard solution and plasma sample: 0.4 $\mu g\,l^{-1}$, urine sample: 60 $\mu g\,l;^{-1}$ analysed at nine consecutive days; slope b from the 5-point-calibration curves as described above). The means$\pm$S.D. for the peak area ratios related to the I.S. at these concentrations were 0.119 ± 0.020 for aqueous standards, 0.110 ± 0.016 for plasma samples and 0.126 ± 0.027 for the urine samples. Hence it follows LOD values of 0.17$\mu g\,l^{-1}$, 0.14 $\mu g\,l^{-1}$ and 21 $\mu g\,l^{-1}$ for these matrices. No significant differences between the LOD values in aqueous standards and plasma or urine matrices were found by Student's t-test ($P = 0.95$). The signal-to-noise ratio at these concentrations were approximately 10:1. Hereby our LOD in plasma samples is lower than this indicated in [22], that is 0.66 $\mu g\,l^{-1}$. Under normal conditions, the detection limit for urine samples was 21 $\mu g\,l^{-1}$. If the urine samples had not been diluted before SPE, it would have been possible to lower the limit to <1 $\mu g\,l^{-1}$. However, this is of no practical significance.

For the limit of quantification (LOQ), calculated with the formulas $y_{LOQ} = y_{LOD} + 3$ S.D.$_{blank}$ ($P = 0.998$) and then $x_{LOQ} = LOQ = 6$ S.D.$_{blank}$/slope b, the following values were obtained: 0.34 $\mu g\,l^{-1}$ for aqueous standards, 0.28 $\mu g\,l^{-1}$ for plasma and 42 $\mu g\,l^{-1}$ for urine samples.

For a medical-diagnostical statement, these practical limits of detection and quantification are fully sufficient, especially since the precision close to the limits is also good.

3.4. Precision

The relative standard deviations (R.S.D.) of the intra-assay precision of the complete method for plasma samples were 2.8% (number of samples, $n = 30$; mean concentration of 5-SCD, mean$_{5\text{-SCD}} = 4.8$ $\mu g\,l^{-1}$) and 3.6% ($n = 30$, mean$_{5\text{-SCD}} = 0.8$ $\mu g\,l^{-1}$), and for urine samples 1.7% ($n = 30$, mean$_{5\text{-SCD}} = 2701$ $\mu g\,l^{-1}$) and 2.1% ($n = 30$, mean$_{5\text{-SCD}} = 337$ $\mu g\,l^{-1}$). These values are superbly low. The R.S.D.s for the inter-assay variability were somewhat higher. The values for plasma samples were 9.4% ($n = 17$, mean$_{5\text{-SCD}} = 12.6$ $\mu g\,l^{-1}$), 9.9% ($n = 17$, mean$_{5\text{-SCD}} = 4.9$ $\mu g\,l^{-1}$) and 18.3% ($n = 15$,

mean$_{5\text{-SCD}}$ = 0.85 μg l^{-1}), and for urine samples 4.7% (n = 10, mean$_{5\text{-SCD}}$ = 307 μg l^{-1}) and 4.0% (n = 10, mean$_{5\text{-SCD}}$ = 2690 μg l^{-1}). For intra- and inter-assays we used the same urine samples but different plasma samples.

3.5. Recovery

For the estimation of the relative recovery, we used plasma spiked with 0.8, 1.6 or 4.0 μg l^{-1} 5-SCD and urine spiked with 40, 160 or 400 μg l^{-1} 5-SCD. Each concentration was measured twice in three series (n = 18 for each matrix). For plasma samples, we found a relative recovery of 103.5% (R.S.D. = 11.4%) and for urine samples, it was 105.4% (R.S.D. = 8.2%).

The absolute recoveries for aqueous standards for 5-SCD was 48.2% (R.S.D. = 14.5%), for D-CD 52.2% (R.S.D. = 19.0%) and for αMD only 7.2% (R.S.D. = 20.6%) and thereby fairly low.

But inspite of these relatively low absolute recoveries, we think this method is reliable. Firstly, the entire method is precise and sufficiently sensitive; and secondly, the correctness of the results may be assumed. This is the case because the measured 5-SCD concentrations were in a similar range as described in the literature; in addition, the results for healthy controls and melanoma patients in various stages fell within the expected ranges. Furthermore, the relative recoveries were acceptable and finally the certified values of the commercial plasma control material were confirmed.

3.6. Method development

In the development of our own method, we immediately preferred an extraction method based on the chemical reactivity of the cis-diol group, as such a method has the greatest selectivity. An alumina batch extraction technique appeared unsuitable: an automation of this method as well as a protection of the samples against light and air is not possible and furthermore the detailed history of each sample in a series is different. An optimization of the column switching technique with an affinity column offers only restricted possibilities for variation of conditions.

We obtained good chromatographic separation with an easy two-eluent system, without ionic-pair reagents, that allowed for an isocratic separation of interesting substances. The second eluent was only used for a quick flush of additional peaks.

For the isolation of 5-SCD on the PBA-SPE-cartridges, we tested some buffer compositions, buffer concentrations and pH values. The best results of recovery and selectivity were obtained with Tris buffer pH 8.30, compared with buffers, containing phosphates, acetates or citrates.

The greatest influence on recoveries was found to be the wash step. The application of water and especially methanol (for extraction of catecholamines possible without significant decrease of recoveries) led to a strong decrease of the absolute recoveries.

For the elution step, some diluted acids and acidic buffers were tested, but these showed only minor differences in recovery and selectivity. The optimum elution volume in regard to reproducibility and peak size seemed to be a volume of 1 ml 0.3 *M* HCl.

The used two-channel electrochemical detection was very advantageous to easy check peak purity. Through the chosen relatively low potentials, only few peaks of interference were visible. In spite of the now greater necessary sensitivity range of 2 nA/FS, the baseline showed no stability problems.

The ASPEC XLi with X-tray software, with its comprehensive possibilities of configuration and the multifarious adjustable parameters to meet the desired requirements, was very suitable for the determination of 5-SCD.

3.7. Investigations of stability of the 5-SCD concentration in plasma

We investigated the stability of 5-SCD plasma concentrations during extraction process under various conditions of temperature, light and air–oxygen (intra-assay series) and compared the suitability of two internal standards (D-CD versus αMD). For these purposes we used spiked plasma (see Fig. 3). The 5-SCD concentrations in each series are different from another.

Generally, the values for R.S.D.s in most of these intra-assay series ranged from low to just acceptable, also under non-optimal conditions. The best results for reproducibility for both tested internal standards

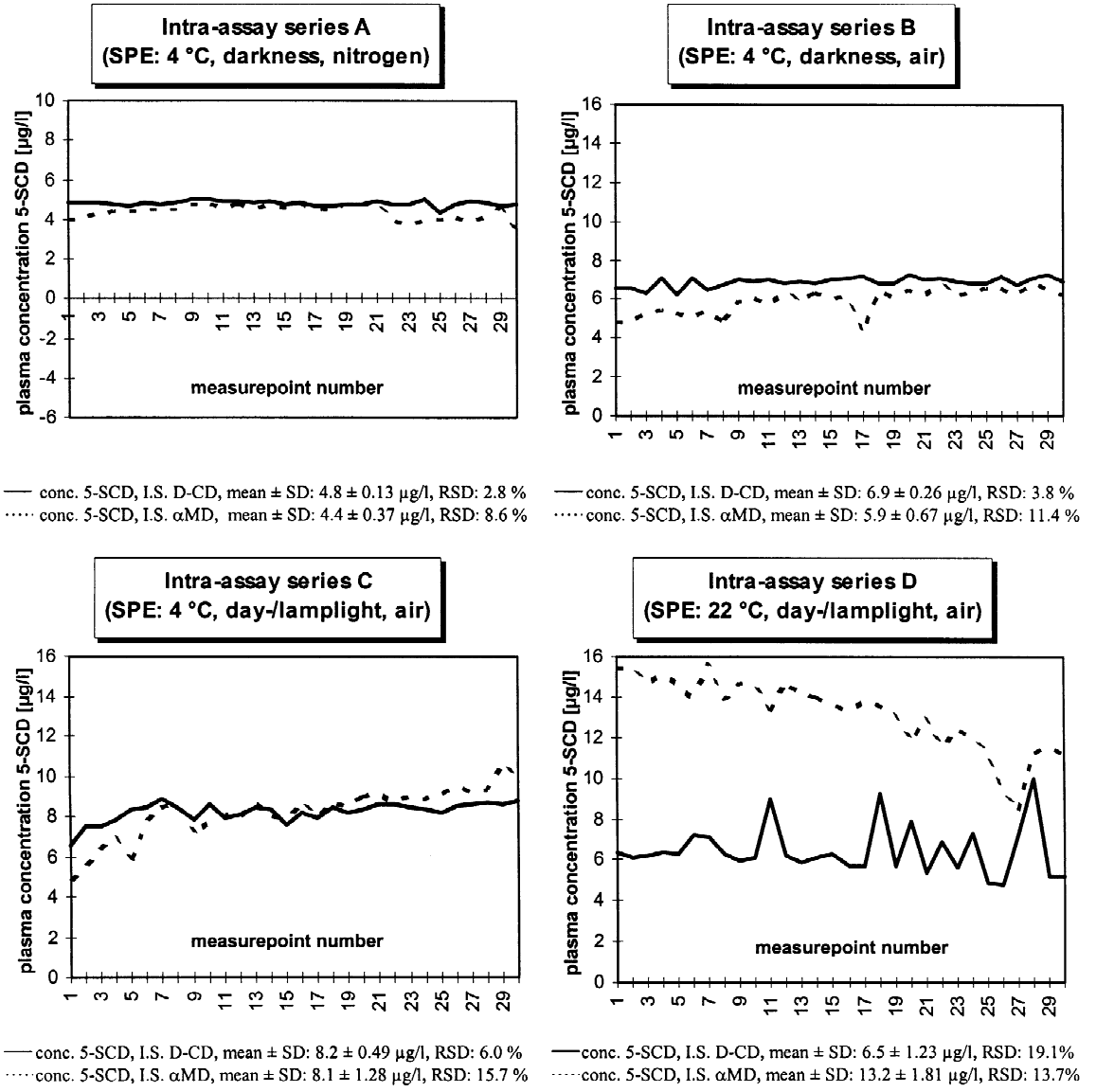

Fig. 3. The precision, correctness and course of 5-SCD concentrations in the intra-assay series A–D in plasma samples (a) after extraction processes with various conditions of temperature (b), light (c) and air–oxygen (d) and comparison of the suitability of two internal standards. (a) In each of the series A–D another plasma was used; (b) temperature of ASPEC; (c) ASPEC racks are protected from light or not; (d) pressure gas, used for complete passage of solutions during SPE on the ASPEC.

were obtained when the protection measures were at its best, that is sample cooling, light protection and use of an inert gas as pressure gas. Lessening of this protection led to worse precision and thereby to unreliable 5-SCD concentrations and faulty medical-diagnostical statements. These facts are shown very clearly in Fig. 3, which presents the course of 5-SCD concentrations within the different series.

From both I.S., D-CD is the more suitable one than the often used αMD. As can be seen in Fig. 3, we obtained for 5-SCD concentrations, when calculated with αMD, a movement within the series even under

optimal extraction conditions. The measured mean 5-SCD concentrations were different as to the applied I.S., whereby the similar values in series C should be coincidental. This shows that αMD poorly reflected the conditions for 5-SCD in the samples and thereby αMD is completely unsuitable as an I.S.

The importance of an immediate injection of the SPE-eluate into the HPLC system is clearly shown in Fig. 4. It can be seen that the 5-SCD catabolism in the eluate started promptly and this cannot be corrected through an internal standard. The great difference in 5-SCD concentrations for both I.S. will not be discussed at this point. Merely after one hour in a autosampler under normal conditions of room temperature and light, the concentration of 5-SCD in the extraction eluate decreased from pathological to border/normal range.

With respect to the high sensitivity of 5-SCD for decomposition, we investigated some parameters of the pre-analytical phase. One of the main questions was whether a decrease of 5-SCD concentration would occur after a waiting time between blood collection and centrifugation. The expected outcome, a decrease in 5-SCD concentrations, actually took place. The absolute and relative decrease in 5-SCD plasma concentrations after a 3–4 h waiting time at 4°C in the dark were $-0.85\ \mu g\,l^{-1}$ and −12%, respectively. In eight of 14 cases (57%), we observed a mostly distinct decrease of concentrations after a waiting time, in five of 14 cases (36%) the concentrations were constant. The latter results were found without exception in cases of low concentration. We only measured in one case a slightly increase in concentration after the waiting time; this might be due to a deviation of the method. All in all, it must be concluded that the decomposition of the 5-SCD started immediately after the blood collection and it only could be stopped by a prompt centrifugation and freezing of the plasma. This catabolism is not negligible and because of its dimension, it can easily lead to faulty interpretations in the case of in reality slightly pathological and border range patients.

We also investigated the influence of storage temperature and storage time on 5-SCD in plasma samples. We found no significant differences for 5-SCD concentrations in the samples at −20°C and −80°C, respectively, which were analysed eight times within two month (data not shown).

3.8. Measurements of 5-SCD in samples of melanoma patients, patients with other skin tumors and healthy controls

Plasma samples of 20 healthy controls (10 females, 10 males) were measured. All 5-SCD

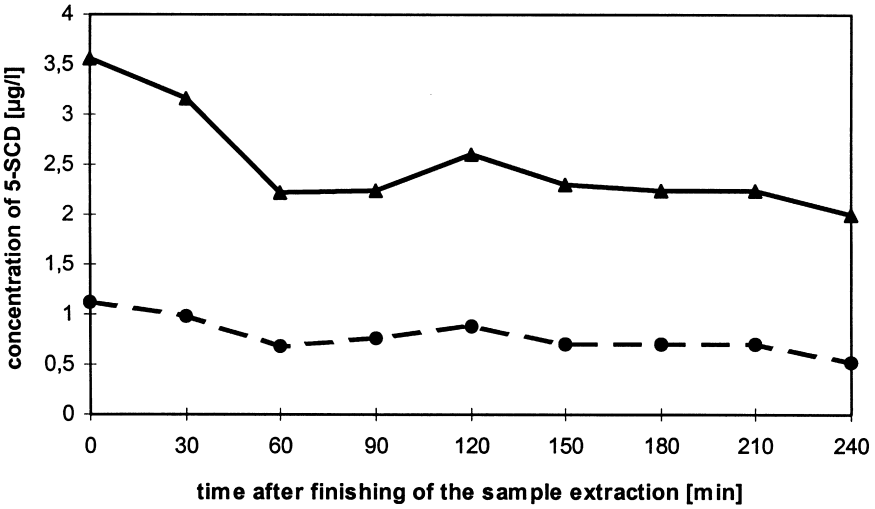

Fig. 4. Change of 5-SCD-concentration of a plasma extraction eluate in a normal autosampler (room temperature, day-light) during repeated injection (chromatographic runtime: 30 min).

concentrations were in the normal range (<1.6 $\mu g\,l^{-1}$). The mean of the male controls was 1.0 $\mu g\,l^{-1}$ (values between 0.6 and 1.4 $\mu g\,l^{-1}$, S.D. 0.28 $\mu g\,l^{-1}$), that of the females was 0.85 $\mu g\,l^{-1}$ (0.5–1.3 $\mu g\,l^{-1}$, S.D. 0.25 $\mu g\,l^{-1}$). These values show a good correlation with some normal values in the literature (0.38–2.84 $\mu g\,l^{-1}$, overview in [12]), partially they are distinct lower.

We investigated 27 patients (14 females and 13 males) with skin tumors other than melanoma. All measured 5-SCD concentrations were below the pathological cut-off level of 3.2 $\mu g\,l^{-1}$ (mean 1.3 $\mu g\,l^{-1}$, values between 0.3 and 2.6 $\mu g\,l^{-1}$, S.D. 0.63 $\mu g\,l^{-1}$). 21 of 27 values (78%) were in the normal range, six of 27 (22%) were in the border range (1.6–3.2 $\mu g\,l^{-1}$).

For our first study, we investigated 77 plasma samples of melanoma patients (30 females, 45 males). These patients were in different clinical stages: 25 patients in the stages I and II (primary tumors), 23 patients in stage III (locoregional metastases) and 29 patients in stage IV (advanced metastatic disease). Pathological or border range 5-SCD-concentrations (>1.6 $\mu g\,l^{-1}$) were found for 10% of patients in the stages I and II, for 36% of patients in stage III and for 66% of patients in stage IV. All patients, that were pathological at stage I/II, had a Clark level IV. All four patients (all in stage IV) with brain metastases showed pathological 5-SCD-concentrations.

Horikoshi et al. [11] found that seven of nine patients (78%) in stage IV showed pathological 5-SCD values in serum. In our study 66% of the patients in this stage had pathological or border range 5-SCD concentration. That seems to be relatively low. But, in the study [11] only nine patients were enclosed. Our patient group has a very heterogeneous structure. This means the type, the size and the localisation of the primary tumor, the number and localisation of the metastases, the age of the patients, the period since first diagnosis of the melanoma and also operations and chemotherapy. On the other side the measured 5-SCD concentrations of all this patients correlated very well with their actual clinical profile. By definition a patient in stage IV remains in this stage, independent of the actual clinical situation after operations and or therapies. So we could observe, that all patients in stage IV, that showed nonpathological 5-SCD concentrations, were in 'stable condition' and showed no progress of disease.

We determined the 5-SCD excretion in 24 h urines of eight melanoma patients in different clinical stages. Patient 1 had a pathological value (1134 $\mu g\,day^{-1}$), patients 2, 3 and 4 border range values (338, 331, 182 $\mu g\,day^{-1}$, resp.). In the meanwhile, patients 1 and 2 have died and patient 3 has showed a progress of disease. The other patients had normal 5-SCD excretions (<150 $\mu g\,day^{-1}$).

Altogether 5-SCD is very useful as a prognostic marker for the malignant melanoma.

4. Conclusions

5-SCD in plasma and urine samples was determined with a newly developed method. On the one hand, this method included optimized conditions for blood collection, storage and transport of samples. On the other hand, a new extraction and separation technique required for 5-SCD was introduced. The extreme sensitivity of 5-SCD against oxidation and light, known and taken into consideration from the beginning, was confirmed again and again. It could be shown, that non-optimal analytical conditions can cause false results. From this point of view, it is not surprising that different authors have obtained in the past contradictory results for sensitivity and specificity of 5-SCD. The plasma 5-SCD concentrations of patients with melanomas in various clinical stages, measured with our method, correlated very well with their clinical profile and better than described in literature hitherto.

In comparison to some other published methods for determination of 5-SCD, our method has the following characteristics.

4.1. Pre-analytical phase

(a) use of an oxidation prevention substance during the blood and urine collection;

(b) immediate centrifugation of the blood at 4°C and freezing at −20°C or lower;

(c) thawing of the samples in the dark at 4°C just prior to the analysis.

4.2. Analytical phase

(a) instead of the commonly used manual alumina batch extraction, we used an automatic system with phenylboronic acid SPE cartridges;

(b) immediate injection of the SPE eluates into the HPLC system to prevent decomposition;

(c) conditions for each sample in a series are nearly the same, as the result of (a) and (b);

(d) cooling of all samples which were not in operation;

(e) extensive light protection during extraction procedure;

(f) exclusion of air–oxygen during SPE, but application of the inert nitrogen;

(g) use of the more suitable I.S. D-CD;

(h) minimal sample requirements: 1 ml plasma, 50 μl urine;

(i) easy composition of HPLC eluents with isocratic separation of interesting peaks;

(j) detection of the peaks with a two-channel electrochemical detector at low potentials.

Our method worked almost completely automatically and was fast and stable for a large sample throughput.

In our work, we could in principle confirm the results of Kagedal et al. [25] concerning the stability of 5-SCD in urine samples. Similar investigations could be applied to plasma samples.

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