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Journal of Chromatography B, 764 (2001) 409–443

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Review

Cysteine and indole derivatives as markers for malignant melanoma☆

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Abstract

Malignant melanoma is a skin tumour, which carries a very unfavourable prognosis. The early detection of a melanoma and even more its metastasis is of decisive importance for the survival prognosis of the patients. So there is always a desire for simple, economical and meaningful serological markers. From the cysteine- and indole-related derivatives, 5-*S*-cysteinyldopa (5-SCD) and 6-hydroxy-5-methoxy-indole-2-carboxylic acid (6H5MI2C) are the most important substances for this purpose. For 5-SCD, the sample pretreatment was carried out either by a manual extraction onto alumina, by an automated method onto boronic acid affinity gels or by an automated solid-phase extraction. For 6H5MI2C, liquid–liquid extractions or direct injection techniques were applied. The chromatographic analyses in the early years were mostly performed with GC–MS. Today HPLC is the nearly exclusively used separation technique. For HPLC, standard RP18 separating columns and usual compositions of eluents were applied. As detectors both the ECD and the FD showed a sufficient sensitivity and selectivity. 5-SCD and 6H5MI2C are very sensitive to light and oxidation. These properties must be taken into account in the complete analysis procedure, including the sample collection, otherwise false low values will result especially for plasma samples. For a critical discussion of the analytical methods and still more for the interpretation of the obtained results, the detailed analytical procedures must be considered. 5-SCD in plasma is one of the best markers of malignant melanoma. It shows an excellent specificity and also an adequate sensitivity in the metastatic melanoma stages. For the detection of primary melanomas and for urine instead of plasma samples, the sensitivity of 5-SCD is generally lower. Altogether, the sensitivity of this parameter is not yet sufficient. 6H5MI2C and other indole derivatives have been investigated far less than 5-SCD. 6H5MI2C correlates less clearly with the different stages of the melanoma and is therefore a less suitable marker. To improve the sensitivity of the findings, in future the investigations should be performed as multi-marker analysis with the simultaneous measurements of more than one marker substance in a given patient sample. Not only one measurement should be carried out per patient, it would be more meaningful to observe the patients with laboratory diagnostics in the follow-up.

Keywords: Reviews; Malignant melanoma; Cysteine; Indole

☆This work is dedicated to the 65th birthday of Professor E. Christophers, Head of the Department of Dermatology, University of Kiel.

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00278-X

Contents

1. Introduction

1.1. Definition, epidemiology and aetiology of malignant melanoma

Malignant melanoma is a tumour which is outgoing from the melanocytic cell system and manifests predominantly on the skin. It can rarely be found on the eye (uvea and retina), the meninges and the mucous membranes [1]. In the majority of cases the melanoma is strongly pigmented. It has a distinctive tendency to develop early lymphogenous and haematogenous metastases and carries a very unfavourable prognosis. The malignant melanomas

(only 1–3% of all cancer diseases [2] and 5–11% of all skin cancer diseases, resp.) causes 75–90% of deaths relating to all skin cancer diseases [1,3].

The incidence of the melanoma is increasing worldwide, but this is subtly differentiated to see. Several factors determine the aetiology, of which the skin colour and the sensitivity to solar radiation dominate. Therefore, the fair-skinned groups of the population in strongly sun-exposed geographical areas [4] are primarily endangered. In some ethnic groups like Asians and Africans, the melanoma is a very rare disease. If it occurs, then it is located primarily in the area of the mucous membranes and palmoplantar regions [1]. The incidence of the melanoma in these groups is much lower and has not changed substantially for years.

In the USA, the risk of developing a melanoma has more than doubled in the last 20 years: From one melanoma per 250 persons (1980) to one per 75 persons (2000). In certain regions in Australia and New Zealand, the risk is even one per 30 inhabitants [5–7]. In Europe, there is a clear gradient to be seen with low risk in the southern countries and high risk in the north. The incidence of melanoma ranges from 1.7 per 100 000 of the male population in Greece to 11.5 of the female population in Denmark (EU: 4.6 male, 6.5 female), and there are remarkable differences between men and women [8]. In the German-speaking regions, the median of the manifest diagnosis of a malignant melanoma lies above 50 years [1].

Further important risk factors for the development of malignant melanoma are an inherited autosomal-dominant predisposition (familial atypical multiple mole melanoma syndrome, FAMMM), as well as the existence of a large number of congenital, acquired or dysplastic nevi. It is interesting that the simple cumulative solar radiation does not increase the risk of melanoma formation as much as severe sunburns in the childhood and adolescence years [2]. Further details can be found in Refs. [9,10].

1.2. Classification, diagnostics and therapy of malignant melanoma

One can distinguish clinically and histologically some types of melanoma: superficial spreading melanoma (SMM, 57% occurrence [1]), nodular melanoma (NM, 21% occurrence, bad prognosis), lentigo maligna melanoma (LMM, 9% occurrence), acral lentiginous melanoma (ALM, 4% occurrence, worse prognosis), non-classifiable melanoma (UCM, 4% occurrence) and other melanomas (5% occurrence, including amelanotic melanoma, which is difficult to diagnose due to missing pigmentation).

Some more differentiations are made: according to the Breslow staging on vertical tumour thickness [11], according to the Clark staging on the level of skin invasion [12] and on the occurrence of metastases [1]. According to the staging system of the AJCC (American Joint Committee on Cancer, 1992 [13]) malignant melanomas are differentiated in stage I (localised primary melanoma, thickness <1.5 mm, 5-year survival prognosis (5YSP) 85–95%), stage II (localised primary melanoma, thickness >1.5 mm, 5YSP 50–70%), stage III (regional nodal metastases and/or in-transit metastases, 5YSP 30–40%) and stage IV (subcutaneous or nodal metastases beyond the regional lymph nodes or visceral metastases, 5YSP 5–10%).

Approximately 90% of all malignant melanomas are diagnosed as primary tumours without recognizable metastases. The 10-year survival prognosis is altogether 75–80% [1]. A diagnosis of a malignant melanoma as early as possible can clearly lower the mortality of this disease. The results are specified by means of imaging methods, e.g., dermatoscopy and ultrasound examinations. The clinical diagnosis of a suspected malignant melanoma is confirmed histologically in every case. If necessary, laboratory examinations of suitable melanoma markers are carried out.

The main therapy for primary malignant melanomas without recognizable metastases is the surgical excision. If necessary, excision therapy is followed by radiotherapy, chemotherapy or immunotherapy with interferons or interleukins.

In patients with melanoma stage IV, therapy is primarily palliative because no adjuvant therapy forms with a clear benefit for survival of patients have been found until now. The treatment is focused on improving the time to tumour progression, the duration of overall survival and symptom relief.

There are some promising developments of adjuvant therapies for the future. One of these is the use of different therapeutic vaccines, which could

increase the patient's own immune response to prevent or delay the progress of the disease [14–16]. Another possibility is gene therapy. Cells, which are capable of handling immunomodulatory messenger substances, are injected directly into the tumour tissue. It is hoped that the expression of these substances in the tumour cells stimulates the endogenous immune system, and that the melanoma cells are recognized as stranger cells and destroyed selectively [17].

1.3. Marker for malignant melanoma and their significance

The early detection of a melanoma, and even more its metastasis, is of decisive importance for the survival prognosis of the patients. This means that the correct staging of a patient is the prerequisite for an optimal therapy. For this purpose, besides the direct histological examination of biopsy or operation samples, imaging methods are primarily used. The most important methods are ultrasonography, magnetic resonance imaging, computed tomography, scintigraphy and, recently, also positron emission tomography. These techniques usually have very high sensitivities and specificities. But none is optimally suitable for all clinical cases, particularly for the detection of micro metastases. Most of these methods are very expensive and partly also invasive for the patients. Some reviews of these methods were recently published [3,18].

There is always a desire for simple, economical and meaningful serological markers for malignant melanoma. Some of them have already been established in diagnostics, others are still in the research stage. The comparison of the suitability of these markers is difficult, because different analytical methods, cut-off values, patient groups, melanoma classifications and in some cases also multiple sampling per patient lead to varying results. All of these marker substances show the greatest sensitivity in the advanced stages of the melanoma and have little or no significance in early diagnostics. None of these markers reaches a specificity of 100%. Another aspect is the suitability of the markers in the follow-up of patients. The question is, if the markers can reflect the course of the therapies and the success or failure of therapeutical treatments. Markers can help to find those patients who are therapy non-responders and who can therefore be spared the most aggressive therapies.

Table 1 lists the currently mostly used serological markers. More details can be found in some recently published overviews [19,20].

2. Cysteine and indole derivatives as markers of malignant melanoma

2.1. Metabolism and biochemistry of the melanin formation

The colour of skin and hair in humans and other mammals is primarily determined by the content and the manner of epidermal melanins and is controlled genetically. Melanogenesis, i.e., the formation of the melanins, takes place in the melanocytes [55–58]. These are cells derived from the melanoblasts of neural crest origins. The melanocytes are found mainly in the epidermis and around the hair bulbs. The biosynthesis of melanins takes place in special membrane-embedded organelles of melanocytes, the melanosomes [59]. In these melanosomes tyrosinase, the key enzyme of the melanin formation is found.

Two types of melanin pigments are normally distinguished, the black–brown eumelanins and the yellow–red pheomelanins. They are produced both in normal melanocytes, but also in melanoma cells. Mostly they are found as mixed-type melanins [60].

The synthesis of the melanins [61–66] proceeds in a multi-stage combination of enzymatic and normal chemical reactions. It starts with the tyrosinase-catalyzed hydroxylation of the amino acid tyrosine to dopa. This enzyme also catalyzes the further oxidation of dopa to dopaquinone.

In the pheomelanotic pathway (Fig. 1), from this very reactive intermediate, 5-*S*-cysteinyldopa is directly formed (with other cysteinyldopa isomers in small portions). The prerequisite for this is the presence of cysteine. A side reaction with an indirect method of cysteinyldopa formation (over 5-*S*-glutathionyldopa) has also been recognized [67,68]. The further oxidation of cysteinyldopas leads to cyclization to 1,4-benzothiazines [56]. Finally, these intermediates dimerize to different trichochromes [69,70] or polymerize to the pheomelanins.

Table 1
Currently discussed serological markers of malignant melanoma

Melanoma marker	Abbreviation	Biomedical function	Comment	References
S100β protein	S100β	Acidic calcium-binding protein, e.g., found in glial cells, melanocytes and melanoma cells	Most frequently used marker; clear correlation between serum levels and melanoma stage; very high specificity; in published studies distinct, varying sensitivities were found; for detection of melanomas in stages I and II not successful; the general suitability as a marker of disease progression and for therapy monitoring must still be confirmed by greater patient groups	[19,21–30]
5-*S*-Cysteinyldopa	5-SCD	Metabolite of the pheomelanotic pathway	If analyzed with a suitable analytical method, one of the best markers of the metastatic melanoma; very high specificity	this work
6-Hydroxy-5-methoxy-indole-2-carboxylic acid	6H5MI2C	Metabolite of the eumelanotic pathway	The sensitivity and specificity has not reached that of other markers; reflects more the melanogenesis in normal melanocytes; possibly analytical problems	this work
Tyrosinase activity		Key enzyme of the melanin formation	Only low sensitivity and specificity	[31,32]
L-Dopa/L-tyrosine ratio		Indirect measurement of tyrosinase activity	Interesting to overcome analytical difficulties in 5-SCD determination; only very few data are available	[33,34]
Melanoma inhibitory activity protein	MIA	Autocrine-secreted tumour cell growth inhibitor	A very promising marker; an assessment is difficult since only very few data are available	[25,35–37]
Interleukins	IL-6 IL-8 IL-10	The detailed functions in tumor immunology is not clear; interleukins were	It is interesting that such immunological substances play a role in the melanoma disease;	[38–40]
Soluble intercellular adhesion molecule 1,	sICAM-1	produced by melanoma cells; cell adhesion	some other conditions influencing the immunological system can affect	[41–43]
Soluble vascular cell adhesion molecule 1	sVCAM-1	molecules were expressed on melanoma	the serum levels of these substances; these substance groups still are in the	
Soluble IL-2 receptor	sIL-2R	cells	research state	[44,45]
Neurone-specific enolase	NSE	Enzyme of the glycolytic pathway	Shows a relative low sensitivity and specificity related to other markers; only very few data are available	[46,47]
Lipid-bound sialic acid	LASA-P	Sialoglycolipid, bound to membranes of tumour cells	Shows a relative low sensitivity and specificity related to other markers; only very few data are available	[48]
Cathepsin D		Aspartyl endoproteinase, plays a role in breast cancer	Plasma levels cannot supply reliable prognostic values for developing metastases	[49]
Lactate dehydrogenase	LDH	Standard laboratory parameter	Not a specific marker of the melanoma; increased serum levels reflect the tumour burden and the tumour cell turnover; it is more an independent prognosis factor for tumour patients	[25,50,51]

Table 1. Continued

Melanoma marker	Abbreviation	Biomedical function	Comment	References
Reverse transcription-polymerase chain reaction for detection of tumour cells in peripheral blood	RT-PCR	Amplification of melanoma-specific RNA/DNA sequences (e.g., tyrosinase mRNA) by PCR	An interesting approach, but at present this sensitive method is still showing some methodical problems for this purpose – resulting, e.g., in strongly differing RT-PCR positive study results from 0 to 100%	[52–54]

In the eumelanotic pathway (in the absence of cysteine or other SH-compounds) the oxidation of dopaquinone leads to dopachrome (Fig. 2). The exact conditions of the reactions in the following steps have not been explained in all details yet. The largest quantity of dopachrome is primarily changed by decarboxylation to 5,6DHI. Minor quantities of dopachrome lead to 5,6DHI2C by enzymatic transformation. These two reactions and the role of dopachrome tautomerase have been the aim of

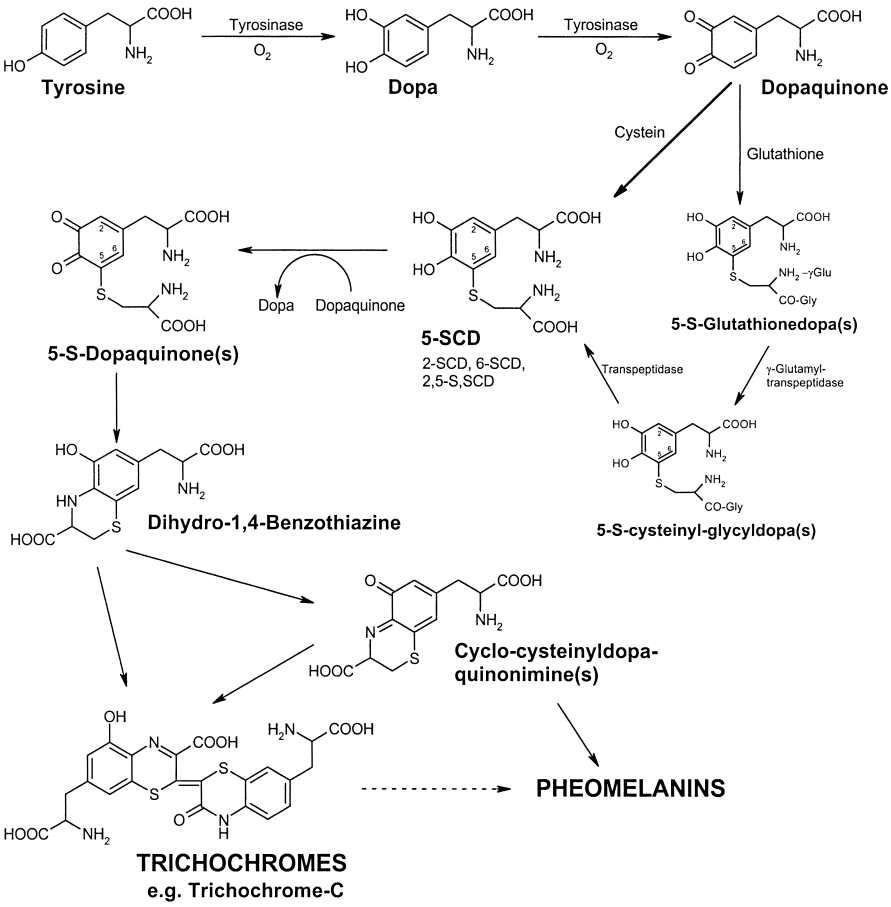


Fig. 1. Metabolic pathway for the synthesis of the melanin pigment pheomelanin.

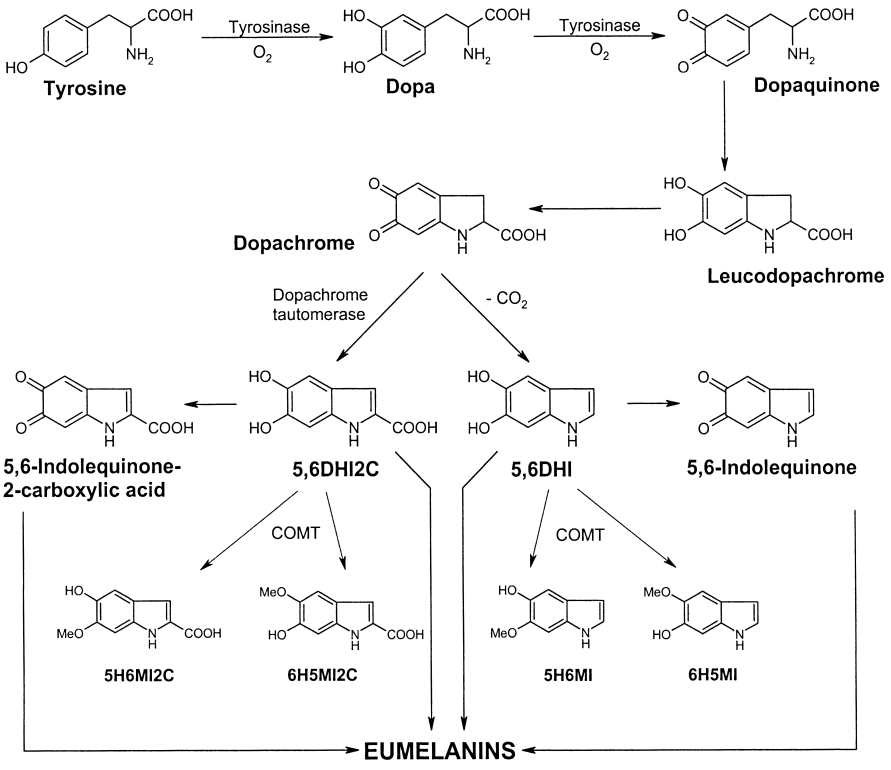


Fig. 2. Metabolic pathway for the synthesis of the melanin pigment eumelanin.

numerous research studies within the last years [71–74]. Both dihydroxyindoles are oxidized further into the corresponding indolequinones, which finally yield the eumelanin pigments by polymerization.

Minor amounts of the cysteinyldopas and the indole derivatives may be leaked into the blood circulation, partly *O*-methylated by COMT in the liver (but COMT also occurs in the melanocytes) and excreted into the urine. Noticeable amounts of these monomers in urine are present as conjugates of the glucuronic and sulphuric acids. This concerns above all the more hydrophobic indole derivatives without carboxylic group and is less meaningful for the indole carboxylic acids [75] and 5-SCD [76].

The balance between the pheomelanotic and the eumelanotic pathway is primarily regulated by the availability of cysteine. Independent of the available quantities of dopaquinone, if cysteine is present in the melanocytes, pheomelanin formation dominates [77].

2.2. Clinical importance and history of melanoma-related cysteine derivatives

The early work of Prota and co-workers [78,79], in which they suggested that pheomelanins are deduced from tyrosine and cysteine and the structure elucidation of 5-SCD by the same authors, was the beginning of the biochemical research in this field. 5-SCD was first found in tissue samples of melanoma cells [80,81], then in urine samples of melanoma patients [82,83] and normal persons [84,85], later also in serum [86,87].

5-SCD is the most important isolated intermediate product in the pheomelanotic pathway. 2-SCD, 6-SCD and 2,5-S,SCD were found to be by-products. The isomeric ratios 5S:2S:6S:2,5S,S were approximately 75:15:1:5%. These ratios were obtained not only in melanoma cells and urine of melanoma patients, but also in the enzymatic synthesis of the cysteinyldopas in vitro [88–90].

5-SCD reflects the tyrosinase activity of normal and pathological melanocytes or melanoma cells [68,91]. No significant differences in the 5-SCD content were seen between the different kinds of primary melanomas [92]. There is an increased excretion of 5-SCD in the urine of melanoma patients, but also a strong dependence on the stage of the melanoma, i.e., if metastasis occurred [93–96]. Ninety percent of 571 patients with metastatic melanomas, but only 9% of 410 patients with melanomas without metastases showed increased 5-SCD concentrations in urine [97]. Not all authors found such optimistic results [98]. Hara et al. [92] observed pathological 5-SCD excretions in urine only in 14% of 43 patients in stage III and in 62% in stage IV. Also, for the correlation of the 5-SCD urine excretion with the melanoma tumour mass, contradicting results were found [99–102].

By measurements of 5-SCD in serum or plasma the sensitivity of this marker could be improved. In serum/plasma a correlation was found between 5-SCD concentration and tumour size in melanoma-bearing mice [103,104]. 5-SCD in plasma reflects the metastasis of the melanoma far earlier and more sensitively than in urine [98,105–107]. Plasma gives some more direct information about melanogenic activity. Urine is influenced too strongly by metabolic processes like oxidation, *O*-methylation and conjugation. However, also in serum/plasma samples, but not in all of the patients with melanoma, not even in everyone in the stages III and IV, increased 5-SCD concentrations were found [98,107]. With our measurements in plasma, we found border range or pathological 5-SCD concentrations (>5 n*M*/l) in 10% of 25 patients in stages I and II, in 36% of 23 patients in stage III and in 66% of 29 patients in stage IV [108].

The occurrence of 5-SCD in urine and in the serum of healthy normal persons must be seen separately from malignant melanoma. 5-SCD is found also in urine and in the serum of healthy normal persons. The 5-SCD excretion and serum concentration of normal persons [109] are independent of skin type and colour of hair, in contrast to the indole derivatives [110]. 5-SCD is also found in the plasma of patients with oculocutaneous albinism or vitiligo, where the 5-SCD concentration was approximately in the same range as in normal persons [105,110–112]. This means that the 5-SCD production is not restricted to the tyrosinase-dependent reaction in the melanocytes, but there are further sources [107,113,114]. 5-SCD in serum of normal persons shows seasonal fluctuations. In summer the values are twice those in winter. However, these values usually do not exceed the normal range of <10 n*M*/l and correlate with the intensity of solar radiation [109,115]. Therefore, an increase of 5-SCD in serum and urine after UV stimulation was found [100,116–118].

An assessment of the significance of 5-SCD as a marker of malignant melanoma is not free of contradictions and therefore not easy to determine.

(i) 5-SCD concentration in plasma is, besides S100β, one of the best markers of the metastatic melanoma. 5-SCD is less suitable if it is measured in urine and if it is used for recognition of primary melanomas without metastasis, because the sensitivity strongly depends on the stage of the melanoma patients.
(ii) 5-SCD in plasma relatively quickly detects changes in the status of the patients, e.g., after an operation or successful therapy treatment [44,119]. Usually the 5-SCD concentration decreases into the normal range within 3–5 weeks.
(iii) 5-SCD shows an excellent specificity of almost 100%. Other skin diseases and tumours do not influence this parameter, neither in urine nor in plasma [92,108,120]. Only persons with many congenital or dysplastic nevi or ‘giant pigmented nevi’ sometimes show increased 5-SCD values.
(iv) With regard to the sensitivity of this marker, and to the normal values found over the years, contradictory results exist, particularly for serum/plasma measurements. A substantial reason for this surely resulted from improper analytical conditions. The oxidative sensitivity of 5-SCD in pre-analytics and analytics frequently was not sufficiently taken into account. The results were false low values [108]. Furthermore, studies on 5-SCD in serum or plasma have often included only a limited number of patients.
(v) The calculated percent quotas for normal and increased 5-SCD values are strongly dependent on the homogeneity of the respective patient

groups, which are often very heterogeneous. This refers, e.g., to the type, the size and location of the primary tumour, to the number and location of the metastases, to the age and sex of the patients, to the time period since first diagnosed and also to operations carried out as well as medication. By definition, a patient who has reached a certain stage remains in this stage, independent of the actual clinical situation after operations and/or therapies. Therefore, not every patient in stages III and IV has metastases and/or elevated 5-SCD values.

(vi) Despite numerous trials with sophisticated statistical methods [23,96], correlation with the location of the metastasis [24,121] or with standard laboratory parameters [22,122], only minor improvements in the sensitivity for 5-SCD could be obtained.

Finally, in this section, some new interesting works should be mentioned. Ros-Bullon et al. [31] did not examine 5-SCD as a product of metabolism of the tyrosinase activity, but the activity of the serum tyrosinase directly. The mean values for patients with melanoma and for normal persons are significantly different from each other, but an explicit differentiation between these two groups is not possible. An overview to this topic is found in Ref. [32].

Letellier et al. [33,34] did not examine the activity of the tyrosinase in plasma directly, but did this via the L-dopa/L-tyrosine ratio. This ratio significantly distinguishes patients with metastatic melanomas from normal persons. But this method is not suitable for an early diagnosis of a primary melanoma.

2.3. Clinical importance and history of melanoma-related indole derivatives

The spontaneous darkening of urine on air and the simple colorimetric reactions by Thormählen [123] and von Jaksch [124] were the only biochemical methods to detect a metastatic malignant melanoma until the 1960s. Clinical studies remained focused for a long time on these so-called ''Thormählen positive substances'' [125]. This test is positive for indole derivatives, non-substituted at the 2- and 3-position. With the identification of the structure of these substances early in the 1980s, a strong development started in this biomedical field. The ''Thormählen positive substances'' A, B and C could be identified as 5H6MI-glucuronide, 6H5MI-6-*O*-sulphate and 5,6DHI-6-*O*-sulphate, with little portions of other isomers [126–129].

The group of eumelanin-related indole substances consists of seven compounds (see Fig. 2). The eighth possible substance, 5,6DMI, to our knowledge has not been found yet. All substances could be detected in normal urine: 5,6DHI, 5H6MI, 6H5MI, 5,6DHI2C [130], 6H5MI2C [131], 5H6MI2C [132], 5,6DMI2C [133] and in urine samples of patients with melanoma [75,132,134,135]. Not all of these substances are suitable in a similar manner for analysis. 5,6DMI2C could be found in urine only in extremely low concentrations. Moreover, the differences in the excretions of normal persons and melanoma patients are very low [135]. Some substances are so reactive that the processes of oxidation and polymerization during sample collection and analysis cannot be stopped. This applies primarily to 5,6DHI2C and 5,6DHI [75,118]. 5,6DHI and also the two *O*-methyl derivatives in urine are found mainly as conjugates of the glucuronic and sulphuric acids [136]. The excretion of free 5H6MI in urine is similar to the one of 5H6MI2C. It shows, however, a greater biological variability in comparison with the carboxylic acid derivatives [110].

This leaves the two partially *O*-methylated derivatives 5H6MI2C and 6H5MI2C. These show a relative stability, and the concentrations in the examined matrices are sufficiently high. Of these two isomers, 5H6MI2C is found in a little higher concentration compared to 6H5MI2C. This ratio was found both in urine and in extracts of melanoma cells [137]. Both isomers run parallel in their concentration, so the analysis of one substance could suffice [135].

Thus, 5H6MI2C and/or 6H5MI2C were generally used in investigations and studies. 6H5MI2C reflects the melanogenesis in normal melanocytes in skin and hair well [110,138,139], i.e., the excretion of 6H5MI2C in urine is greater with the genetically determined darker skin types (III, IV) than with the fair-skin type (II). No 6H5MI2C has been found in the urine of albino patients and albino mice [118]. Presumably a seasonal dependence of the excretion of 6H5MI2C exists. This has been found in serum,

but it is still weaker than by 5-SCD and does not correlate with the intensity of solar radiation [115].

No correlations between 5-SCD and 6H5MI2C have been found with the different skin types, either in urine excretion or in serum concentration [105,118].

Both single 5H6MI2C and the sum of 5H6MI2C+ 6H5MI2C in urine and serum correlate very well with the tumour mass of B16 murine melanoma [101,102]. Wakamatsu et al. confirmed this observation for urine but not for serum [104].

In a study, 77 random spot urines of 91 melanoma patients in all stages (85%) had a normal excretion of 6H5MI2C but this did not correlate with the prognosis of the patients [23]. In another study, only 52% of the melanoma patients in stages III and IV had increased random spot urine excretions of 6H5MI2C (5SCD: 83%). This did not show a correlation with the survival rate [96]. The 6H5MI2C concentration in the serum exceeded the normal values only in three of nine melanoma patients with metastases and therefore does not reflect the progression of the melanoma [107]. On the other hand, Hara et al. found that plasma 6H5MI2C correlates very well with the stage of melanoma patients and with tumour thickness, much better than 5-SCD [120].

The melanoma-related indole derivatives were not investigated as frequently as 5-SCD, and of the relatively few studies most were carried out for urine samples and with mostly quite low sample numbers. Systematic investigations, also with hydrolyzed urine samples, are missing.

Altogether, the suitability of 6H5MI2C, as the most important indole substance as a marker of malignant melanoma seems unquestionable. This is because increased values of this compound in urine and plasma of melanoma patients have been found. The elevated levels are dependent on the stage of the melanoma, i.e., the metastasis. But the specificity as well as the sensitivity has not reached that of other markers, especially 5-SCD. And some contradictory results have been obtained. Probably one reason for this is that 6H5MI2C is extremely sensitive to oxidation, even more than 5-SCD (unpublished result in Ref. [105]). This sensitivity has not been mirrored in the applied procedures for pretreatment and analysis.

The renal clearance of 6H5MI2C shows a very great variability. One reason for this could be that the indole derivatives in urine are very dependent on the conditions of conjugation [136]. 6H5MI2C and other *O*-methylated derivatives are formed in melanocytes or hepatocytes by COMT [140]. Therefore, the activity of COMT can have a significant influence on their turnover.

2.4. Reference values of some melanoma-related cysteine and indole derivatives

Table 2 presents a survey of normal values and some pathological values of the discussed substances. For many of these compounds seasonal variations are known or expected. No evaluation was carried out for analytical methods. Sex-dependence of 5-SCD and 6H5MI2C excretion, although discussed, could not be significantly confirmed [97,141]. This also concerns a suspected age-dependence [85,105]. Normal values for 5-SCD and 6H5MI2C for children in random spot urine can be found in Ref. [142].

3. Analysis in the clinical laboratory

For the determination of substances in biological materials and also in cases of melanoma-related cysteine and indole derivatives, a sample pretreatment precedes the actual analysis. This contains the removal of disturbing matrix substances, usually proteins and the enrichment of trace components or both, respectively.

The concentrations of the melanoma-related cysteine and indole derivatives are relatively low. This means, firstly, that a sufficient analytical sensitivity is required. However, modern chromatographic systems have this required sensitivity, at the latest with the use of MS detection. Under these prerequisites, it seems even more important to reach a sufficient analytic specificity, so that clear and correct findings can result — especially if it is such a sensitive parameter like a tumour marker.

However, the ‘‘correctness’’ of analytical results includes even further criteria: is the specimen the right sample material with the right additives? Has the material been obtained, transported and stored

Table 2
Normal values and some pathological values of melanoma-related cysteine and indole derivatives

Substance	Matrix	Concentration/excretion		Comments/references
5-SCD	Urine (NP)	<150 μg/die	Normal range	Japanese subjects [92]
		150–400 μg/die	Border range	Swedish subjects [97]
(M.W. 316 g/mol)		>400 μg/die	Pathological range	
5-SCD	Urine (NP)	Mean: 142 μg/die	⇒mean: 450 n*M*/die	*n*=33, Japanese subjects, late autumn [105]
5-SCD	Urine (NP)	Mean: 296 μg/die	Maximum in August	*n*=10, Japanese subjects [143]
		Mean: 141 μg/die	Minimum in February	
5-SCD	Urine (NP)	Mean: 87 μg/die	⇒mean: 275 n*M*/die	*n*=76, Swedish subjects, autumn [85]
5-SCD free	Urine (NP)	89±79 μg/die	(mean±SD)	*n*=45, [76]
5-SCD total		230±106 μg/d	(mean±SD)	Enzymatic hydrolysis with
ratio total/free	Urine (NP)	2.58		glucuronidase+arylsulfatase
ratio total/free	Urine (MP)	1.36±0.29	(mean±SD)	*n*=10 [76]
5-SCD	Urine (NP)	<70 μ*M*/M creatinine		*n*=71, Swedish subjects [96]
5-SCD	Plasma (NP)	<5 n*M*/l	Normal range	*n*=20, German subjects [108]
		5–10 n*M*/l	Border range	
		>10 n*M*/l	Pathological range	
5-SCD	Serum (NP)	Mean: 4.3 n*M*/l		*n*=33, Japanese subjects, late autumn [105]
		<10 n*M*/l	Normal range	
5-SCD	Serum (NP)	Mean: 8.9 n*M*/l		*n*=20, Swedish subjects [87]
5-SCD	Plasma (NP)	1.2±0.9 n*M*/l	(mean±SD)	*n*=31, Japanese subjects [144]
5-SCD	Plasma (NP)	Mean: 12 n*M*/l	Maximum in June	*n*=9, Scottish subjects [109]
		Mean: 6 n*M*/l	Minimum in December	
5-SCD	CSF (NP)	Not detectable		
	CSF (MP)	160 μg/l	(brain metastases)	*n*=1 [145]
5-*S*-cysteinyl-dopac	Urine (NP)	20±9.1 μg/die	(mean±SD)	*n*=12, Swedish subjects [146]
6H5MI2C	Urine (NP)	Mean: 38 μ*M*/*M* creatinine		*n*=10, Swedish subjects [131]
(M.W. 207 g/mol)		(range 6–76 μ*M*/*M* creatinine)		
6H5MI2C	Urine (NP)	390±320 n*M*/die	(mean±SD)	*n*=33, Japanese subjects, late autumn [105]
(6H5MI2C + 5H6MI2C)	Urine (NP)	890±270 n*M*/die	(mean±SD)	*n*=9, Japanese subjects [95]
6H5MI2C	Serum (NP)	3.6±1.8 n*M*/l	(mean±SD)	*n*=33, Japanese subjects, late autumn [105]
5,6DHI2C	Urine (NP)	11–41 n*M*/l		*n*=3 [147]
	Urine (NP)	28 n*M*/l (5.4 μg/l)		[148]
	Urine (MP)	16.6 μ*M*/l (3200 μg/l)		*n*=1 [147]
5H6MI2C	Urine (MMP)	17.9 μ*M*/l		*n*=1 [136]
6H5MI2C	Urine (MMP)	5.3 μ*M*/l		*n*=1 [136]

Table 2. Continued

Substance	Matrix	Concentration/excretion	Comments/references
5H6MI2C-ester-glucuronide	Urine (MMP)	332 μ*M*/l	*n*=1 [136]
5H6MI2C-5-*O*-sulphate	Urine (MMP)	7 μ*M*/l	*n*=1 [136]
6H5MI2C-ester-glucuronide	Urine (MMP)	199 μ*M*/l	*n*=1 [136]
6H5MI2C-6-*O*-sulphate	Urine (MMP)	27.5 μ*M*/l	*n*=1 [136]

NP, healthy normal persons; MP, melanoma patients; MMP, patients with metastatic melanoma (if it not noticed otherwise, the compounds were analyzed without acid or enzymatic hydrolysis).

correctly? Have necessary required restrictions, e.g., diet or medication been adhered to by the patient? And if not, which influences does this have on the measured result? Unfortunately, these side conditions frequently do not get the attention they should. They can falsify the actual measurement result significantly and so these conditions often have a co-decisive importance for the interpretation of the results. For the laboratory, such conditions are either difficult or only partly possible to quantify. Therefore they should be seen as an integral part of an analytical method.

3.1. Sample collection

From the chemical and biomedical point of view, the melanoma-related cysteine and indole derivatives are extremely sensitive to light and oxidation. Besides this more verbal statement by many authors, also systematic examinations of these properties were carried out. This statement has been confirmed and detailed information has been received [108,149–152]. This requires that the sensitivity must be taken into account in the complete analytical procedure, to avoid false interpretations of the measured values.

3.1.1. Urine

There are two possibilities for sampling, the use of random spot urine (related to the creatinine concentration) or collection of 24-h urine. For many laboratory parameters, like the catecholamines and their metabolites, it is usual to collect 24-h urines, because the biological variability in 24-h urines seems to be the lowest. So generally, it can be recommended to collect 24-h specimens of urine. Independent if random spot urines [23,96,142] or 24-h urines [92,100,111] are used, precautions have to be met, so that the analyzed substances are not oxidized or decomposed before the analysis is performed. Kagedal et al. published a systematic investigation on the stability of 5-SCD and 6H5MI2C in urine at different collecting and storage conditions [152]. The most suitable proved to be the addition of acetic acid to a pH value of 3–4, with or without addition of sodium disulfite and storage at −20°C (see Table 3).

Baars et al. [153] observed an interesting effect on the stability of 5-HIAA. He found in urine acidified with HCl to pH values <2, significant quantities of 5-HIAA will be destroyed. It has to be expected that also other indole derivatives behave similarly.

Generally, the following conditions for collecting a 24-h urine can be recommended: (i) light protection, (ii) addition of 50 ml acetic acid and 1 g sodium disulfite into the 2-l collection vessel immediately after acquiring the first urine portion, (iii) cooling of the urine during the whole collecting period, (iv) after finishing the collection immediate freezing of the aliquot at −20 °C, (v) thawing of the urine in the dark at 4–8°C.

3.1.2. Plasma

In comparison with urine, the concentrations of the melanoma-related cysteine and indole derivatives in plasma or serum are approximately 100 times lower. This means that for plasma analyses improved

Table 3
Stability of 5-SCD ($n=7$) and 6H5MI2C ($n=6$) in urine with different additives: (1) acetic acid to pH 4; (2) sodium disulfite, pH 3–4; (3) thymol/isopropanol; (4) no additives, pH 3–7.5

5-SCD	−20°C				+4 to +8°C				+20°C			
	1	2	3	4	1	2	3	4	1	2	3	4
1 week	95±5	94±5	91±7	92±6	97±3	92±4	75±37	79±36	96±11	91±4	58±37	50±45
2 weeks	99±4	95±4	94±6	92±8	100±6	97±4	73±34	78±40	95±17	94±5	22±33	32±39
3 weeks	96±8	92±11	91±8	86±15	99±10	94±11	69±36	78±47	84±16	87±13	8±20	20±35
4 weeks	101±7	98±8	90±18	88±18	102±15	94±6	62±38	72±48	–	–	–	–
8 weeks	99±7	97±7	87±23	87±16	94±5	91±12	52±38	55±40	–	–	–	–
6H5MI2C												
1 week	98±8	99±10	95±9	89±9	84±7	92±9	88±16	86±10	–	–	–	–
3 weeks	96±10	105±14	86±17	82±16	73±6	97±14	94±25	102±40	–	–	–	–
5 weeks	98±10	104±22	97±8	93±12	69±11	99±19	110±62	116±75	–	–	–	–

Results are given as means±SD (%). From Ref. [152], with permission.

precautions must be taken against degradation of these substances.

The question of whether serum or plasma should be preferred is raised. In clinical practice serum is the material most frequently used, but is formed only slowly. The drawn blood must be left standing for some time. In published works of studies and methods, the sample collection and storage conditions were given only little attention. Serum without additives was usually used for the analyses. After coagulation and centrifugation of blood, sera were frozen (−20 to −80°C). In an early work [87], an oxidation protection substance was added to the serum, but it was stored 3 h at room temperature before freezing.

The key event for us was the 5-SCD analysis of samples in a preliminary study. These 134 serum samples of melanoma patients in the stages III and IV were collected under ''normal'' clinical conditions. That means collection without temperature, oxidation and light protection, thawed and frozen several times. As can be seen in Fig. 3a, only 11 of these samples (8%) showed a measurable 5-SCD peak, mostly only a little over the detection limit. Only one sample had a slight pathological value. In comparison, Fig. 3b shows 5-SCD concentrations of 134 plasma samples of random melanoma patients of all stages, collected and analyzed with consideration for all protective measures [108].

In 1999 we published the results of a systematic examination of parameters influencing the measured values of 5-SCD in plasma [108,154]. It could be clearly shown that not only during sample pretreatment and analysis must the light and oxidation sensitivity of 5-SCD be taken into account, but already in the pre-analytical phase during sample collection. As shown in Fig. 4, a waiting period for EDTA–blood of 3–4 h at 4–8°C in the dark, despite addition of sodium disulfite, led to a clear decrease of 5-SCD concentration in comparison with an immediate centrifugation and freezing of the plasmas (average, −17%, single values up to −40%). The addition of sodium disulfite as an oxidation protection substance here helped marginally. It was not explicitly investigated if other substances (e.g., ascorbic acid, GSH, dithiothreitol [109] or cysteamine) are much more effective for this purpose. In our experience, that should not be expected with certainty. In any case, the addition of an oxidation protection substance is a step in the right direction.

The importance of an immediate injection of the SPE eluate into the HPLC is shown in Fig. 5. After merely 1 h in a normal autosampler, 5-SCD concentration decreased from pathological to border/normal range.

An investigation into the influence of the storage temperature and duration showed no significant differences for 5-SCD in plasma between −20 and −80°C. Eight measurements were carried out at each temperatures in the course of 2 months [108].

A repeated thawing (in the dark at 4–8°C, 5 times within 23 days) and freezing (−80°C) of plasma samples with normal 5-SCD concentrations did not lead to any significant decrease of the 5-SCD values

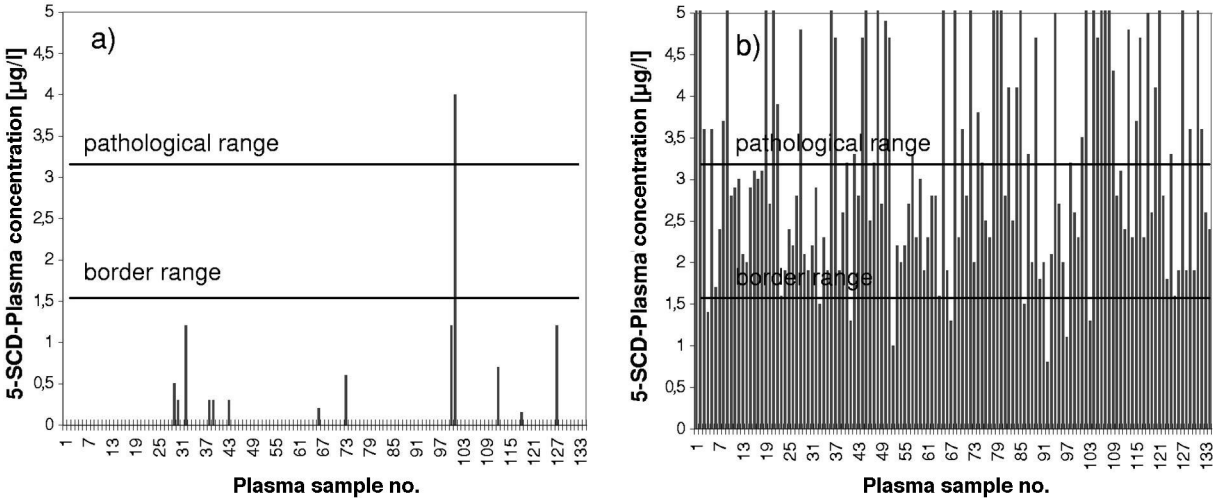


Fig. 3. Significance of pre-analytical parameters for correct results for 5-SCD in plasma. Analysis was performed according to Ref. [108] (from Ref. [154], with permission). (a) 5-SCD concentrations in 134 serum samples from a preliminary study of melanoma patients (stage III and IV), collected under ''normal'' clinical conditions, i.e., without temperature, oxidation and light protection and thawed and frozen several times. (b) 5-SCD concentrations in 134 random plasma samples of melanoma patients (stages I–IV), collected considering all known influence factors.

(J. Hartleb, unpublished results, 2001). No difference, furthermore, could be noticed between the 5-SCD stability in EDTA– and heparin–plasma (each with the addition of sodium disulfite) (J. Hartleb, unpublished results, 2001).

The diurnal and seasonal variations of the 5-SCD concentration in plasma were only low (see Ref. [109] and Section 2.2).

For the blood collection the following procedure can be recommended: (i) blood should be drawn by venipuncture into vacuum tubes containing EDTA or heparin, (ii) immediate centrifugation of the blood, if possible at 4°C, (iii) immediate transportation of the plasma into a new tube containing 100 µl 0.5 *M* sodium disulfite and up to 100 µl of 0.2 *M* EDTA, (iv) immediate freezing of the plasma at a minimum of −20°C, (v) thawing of the samples in the dark at 4–8°C just prior to the actual analysis.

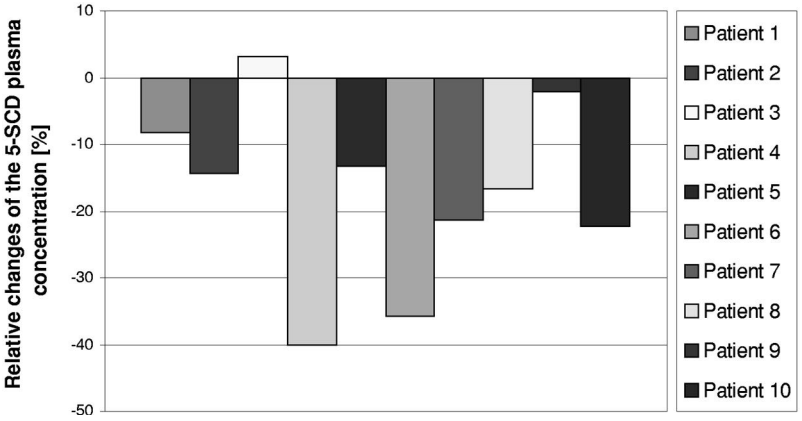


Fig. 4. Relative changes of 5-SCD plasma concentrations of melanoma patients after a 3–4-h waiting time in the dark between blood collection and centrifugation. Analysis was performed according to Ref. [108] (from Ref. [154], with permission).

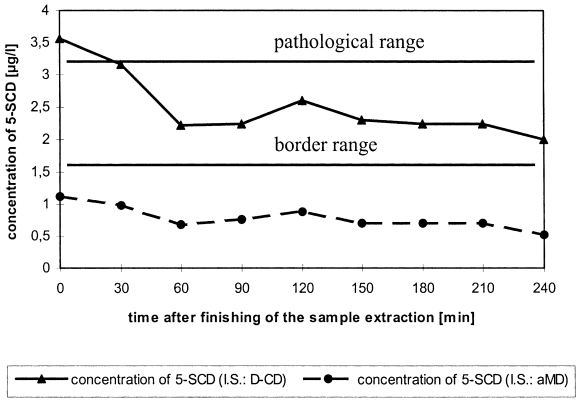


Fig. 5. Change of 5-SCD concentration of a plasma extraction eluate in a normal autosampler (room temperature, daylight) during repeated injection (chromatographic runtime, 30 min) (from [108], with permission).

Until now similar investigations for the indole derivatives in plasma have not been performed. Going from the results for 5-SCD in plasma and for 6H5MI2C in urine [152], it can be concluded that for the protection of the indole derivatives in plasma similar measures are necessary as for 5-SCD.

3.1.3. Cell and tissue samples

Cysteine and indole derivatives in tissue and cell samples are not measured routinely as markers of malignant melanoma. However, measurements in these matrices are very important to elucidate the metabolism of these substances and for characterization of this and other skin diseases. Here a brief summary is given.

Normally, the cell or tissue samples are homogenized (mechanical or with ultrasound) in a 5–10-fold volume of 0.4 *M* $HClO_4$ or HCl and then centrifuged at high *g*-values. The supernatant is partly directly injected into the HPLC system [104,151], partly further processed, as described for urine and plasma samples, and only then it is injected [99,155–157]. In the analysis of biopsy samples of dysplastic nevi with the sample pretreatment technique, described in Ref. [158], Halldin et al. [155] could observe no decomposition of added 5-SCD. However, at this point, the work by Fornstedt et al., who investigated brain samples, should be referred to [157,159,160]. It was observed, auto-oxidation processes and formation of some substances (e.g., 5-cysteinyl-dopamine) can occur in the samples during the sample pretreatment. This was the case, if particular protection conditions were not performed. To the acid used for homogenization (0.1 *M* $HClO_4$), sodium disulfite, ascorbic acid, dithiothreitol and DTPA (not EDTA) had to be added and the complete sample pretreatment had to be carried out as far as possible in an ice bath.

3.1.4. Standards

Details on the stock solutions of the substances and their stability are found only in some publications. This is surprising, considering these compounds are not available commercially (except for diluted working solutions of 5-SCD).

For stock solutions of the cysteine derivatives, preferably of 5-SCD, HCl is almost exclusively used as solvent. The concentration of the HCl was in the range from 0.01 *M* [108] up to 2 *M* [141]. Oxidation protection additives, e.g., 100 mg/l ascorbic acid [161] or 1 g/l sodium disulfite with 1 g/l EDTA [162], are only seldom added. The concentrations of the stock solutions varied from 0.6 m*M*/l [108] up to 2.75 m*M*/l [141]. These 5-SCD solutions, stored frozen, were stable for several months up to 2 years [141].

This relative stability of the frozen stock solutions was not found, however, in the more diluted working solutions, especially not at room temperature. Under these conditions, a relatively fast decomposition of the substances takes place. Interestingly, in Ref. [163] it was found that the addition of 5 m*M*/l sodium disulfite to a working solution of 5-SCD in 0.1 *M* of acetate buffer (with 0.2 m*M*/l EDTA, pH 4.0, room temperature) reduces the stability of the standard.

Good experience regarding the stability of the working solutions were obtained by the use of 1 m*M*/l HCl (with 5 m*M*/l EGTA and 4 m*M*/l GSH) and cooling at 4°C [108]. Ito et al. found, that the hydrolytic stability of 5-SCD was dramatically increased by the addition of 50 mg each of bovine serum albumin (BSA) and cysteamine-hydrochloride [151].

It can be recommended that stock solutions of the cysteine derivatives, with concentrations of approximately 1 m*M*/l in 0.01–1.0 *M* HCl, should become aliquoted in suitable volumes and then frozen. For

working solutions, to avoid hydrolysis, a less acidic pH value (approximately pH 3–4) should be chosen. For each analysis, an aliquot of the stock solution is thawed and diluted freshly and immediately processed, if possible, under cooling. Stabilizing additives to the working solution, like EDTA [141], GSH or cysteamine, are advantageous.

For the indole derivatives, virtually no details on the preparation conditions of the stock and working solutions could be found. Only in Ref. [135] was it published that the different stock solutions in EtOAc were stored at −40°C under nitrogen. A possible reason for the lack of information is that all indole substances must be synthesized and so are available as solids. For the analysis they then become dissolved in suitable solvents, keeping in mind the following sample pretreatment and analysis techniques.

No details of the stability of the stock and working solutions were found, not even for the indole derivatives most frequently used (5H6MI2C and 6H5MI2C). Exceptions are only notes, that 5,6DHI is not stable during enzymatic hydrolysis and in working solutions [135], and that the decomposition of *O*-sulphates of 5H6MI2C and 6H5MI2C in 0.4 *M* $HClO_4$ was less than 5% after 3 h at 25°C [102].

3.2. Synthesis

With only one exception, the here-discussed substances are not available commercially. Therefore the necessity of synthesis exists. So the synthesis methods used are briefly discussed. Only 5-SCD, as a dilute solution (e.g., 20 μg/l), has been commercially available in the last few years (e.g., from Recipe, München, Germany).

3.2.1. Synthesis of cysteine derivatives

The first syntheses of 5-SCD were described by Prota and Nicolaus in 1967 [78]. Here the *N*-acetyl-L-dopa-ethylester was oxidized with silver oxide. The addition of L-cysteine and acidic hydrolysis yielded mixtures of 5-SCD and 2-SCD, which could be separated by column chromatography with cation-exchange resin. These syntheses, however, were quite expensive and 5-SCD was obtained with only low yields (35%) [164]. An improvement of this method led to yields of 70% for 5-SCD [165].

Agrup et al. [88] and Ito and Prota [166] published simple synthesis possibilities with practical quantitative yields by using mushroom tyrosinase: L-dopa reacted with L-cysteine in 0.05 *M* phosphate buffer at room temperature in the presence of tyrosinase. After 30–45 min, the reaction was disrupted by a change in the pH value to pH 1. The purification of the reaction mixture was carried out onto a cation-exchange column and using HCl as eluent. The compounds 5-SCD:2-SCD:6-SCD:2,5-SSCD were yielded in the ratio of approximately 74:14:1:5%. Gradual improvements in this enzymatic synthesis route were later reached [167] by bubbling the reaction mixture with nitrogen, by allowing a distinct longer reaction time (up to 12 h), by changing the pH value slightly (to pH 6.5) [168], by increasing buffer concentration (0.5 *M*) and more tyrosinase activity. Besides 5-*S*-L-cysteinyl-L-dopa (5-SCD), the two diastereomers 5-*S*-L-cysteinyl-D-dopa and 5-*S*-D-cysteinyl-L-dopa (D-CD) were also synthesized in [168]. The importance of D-CD lies in its use as an ''ideal'' internal standard.

Further enzymatic syntheses of related substances, such as 5-*S*-glutathionyl-dopamine [169], 5-*S*-glutathionyl-dopa and 5-*S*-cysteinyl-glycine-dopa [170], 5-*S*-cysteinyl-glycine-dopac [146] and 5-*S*-cysteinyl-methyldopa [162], were published. The latter substance was described also as a very suitable I.S.

Alternative synthesis routes without the expensive tyrosinase were found by Chioccara and Novellino [171]. The oxidation of dopa was performed in the presence of cysteine by means of adding aqueous sulphuric acid and ceric ammonium nitrate under nitrogen. After purification onto a column with cationic exchange material and recrystallization, the yield of 5-SCD was 57% and thus quite high.

It is noted that with the three synthesis methods described in Ref. [165], some other cysteine derivatives were obtained, such as cysteinyl-catechols, cysteinyl-phenols, cysteinyl-histidine, cysteinyl-tyrosine and 5-*S*-cysteamine-dopamine.

3.2.2. Synthesis of the indole derivatives

Synthesis routes for 5,6DHI and 5,6DHI2C and their monomethyl analogues were among others described by Benigni and Minnis [172], however they were relatively expensive. Besides these substances Pavel and Muskiet also prepared the corre-

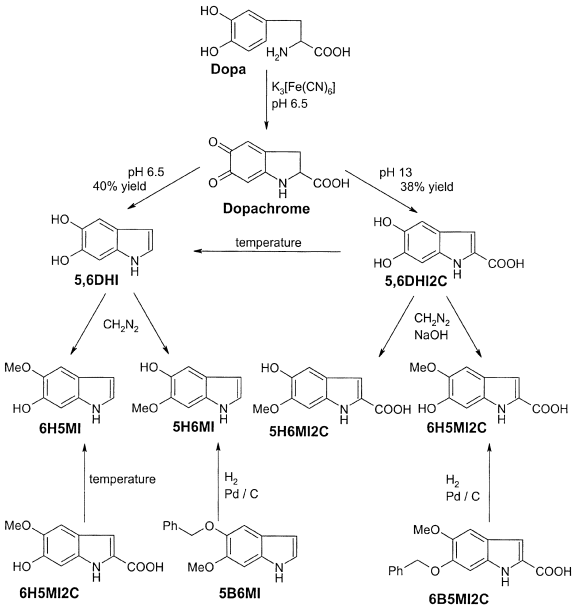


Fig. 6. Synthesis scheme of the preparation of some melanoma-related indole derivatives (from Ref. [174], with permission).

sponding deuterated analogues (standards for GC–MS) [173].

Wakamatsu and Ito criticized an insufficiently detailed description in [173], publishing in 1988 simplified synthesis routes for six substances [174], starting with dopa or with commercially available *O*-benzyl derivatives. The synthesis methods allowed the production of milligram to sub-gram quantities of the compounds. A scheme of these synthesis routes can be seen in Fig. 6.

The preparation of the two dimethoxy derivatives 5,6DMI and 5,6DMI2C was already described in 1927 by Oxford andRaper [175]. Synthesis methods for the preparation of 5,6DHI-5-*O*-sulphate, 5,6DHI-6-*O*-sulphate and 5,6DHI-5,6-O,*O*-disulphate, as well as their methylation products have been described in Refs. [128,129,136].

4. Sample pretreatment

4.1. Sample pretreatment of cysteine derivatives

5-SCD is the most important compound of the melanoma-related cysteine derivatives. It has, like the catecholamines, the amino acid dopa and other substances, two neighbouring hydroxyl groups at the phenyl ring. Since the work of Anton and Sayre in the 1960s [176,177] on the selective adsorption of substances with *cis*-diol groups onto alumina at pH 8.6, this pretreatment and enrichment technique has become a substantial part of the biochemical analysis methods. The unique and slightly accessible selectivity of this reaction, and the simultaneous great stability of the formed complex, is impressive even today and still has an important place in the sample pretreatment techniques. Also later developments, like modified boronic acid derivatives on affinity gels and on silica in SPE materials, work on the same principle.

The number of variants in the sample pretreatment of 5-SCD and similar substances is small. It is not so important from which matrix the sample pretreatment is carried out. The observed effects and influences refer more to the pretreatment medium than to the biological matrix. Unless otherwise mentioned, a HPLC separation always followed the pretreatment procedure.

4.1.1. Sample pretreatment with alumina

The early extractions for the determination of 5-SCD from the years 1972/73 [80,81] were performed with the method by Anton and Sayre [176,177]. The recovery of 5-SCD after this extraction method (followed by fluorometry) was only 37% for urine [84] and 55% for cell extracts [81]. Using this pretreatment technique, plasma samples were also investigated. But 5–10 ml plasma were needed for each analysis and the extraction preceded a protein precipitation. The recovery for 5-SCD was not sufficient, a value of 31% was given (71% for dopa and 81% for the I.S. isoproterenol).

For the improvement of the specificity of the sample pretreatment, the urines were pre-cleaned on a short column with a strong cation-exchange resin before the alumina extraction. As can be seen in Fig. 7, a substantial cleaner chromatogram was then obtained [141]. The relative recovery was 105%, the absolute one was not given. As I.S. a tritium-labelled 5-SCD was used. Problems with the stability of the extracted samples in the autosampler could be solved by the addition of 20 m*M*/l EDTA to the desorption acid.

Ito et al. published in 1984 an extraction method [144] for 5-SCD in plasma, urine and tissue samples,

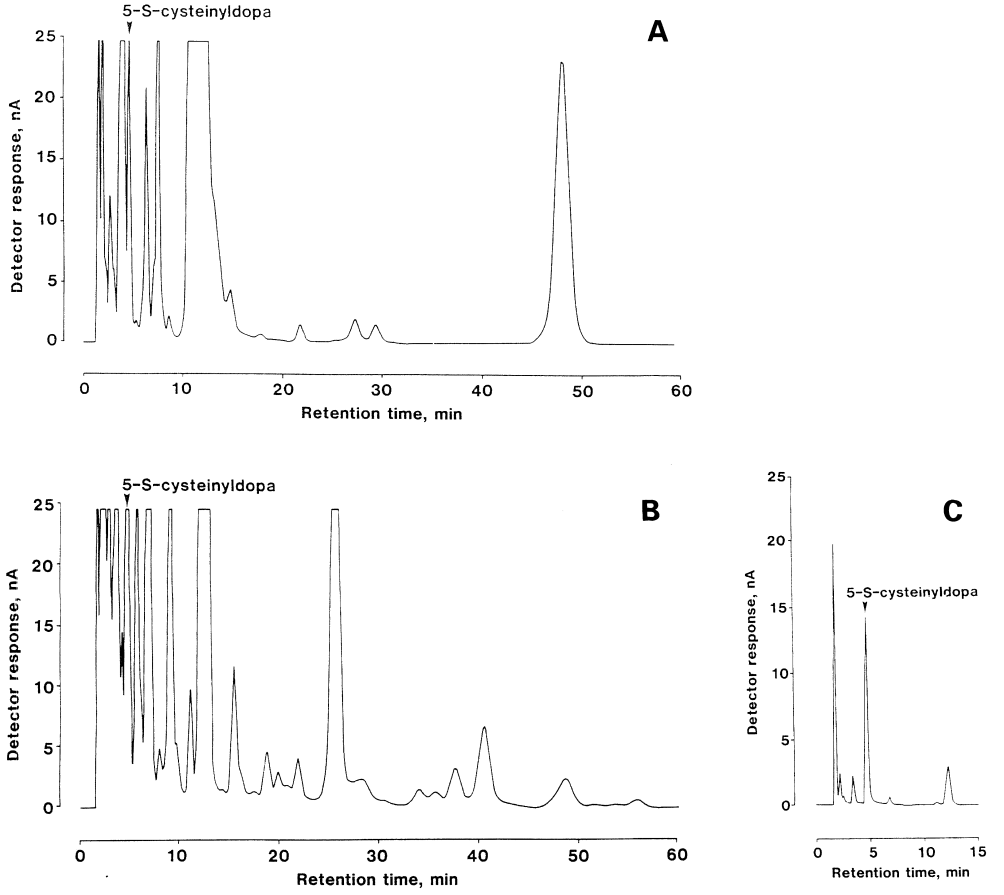
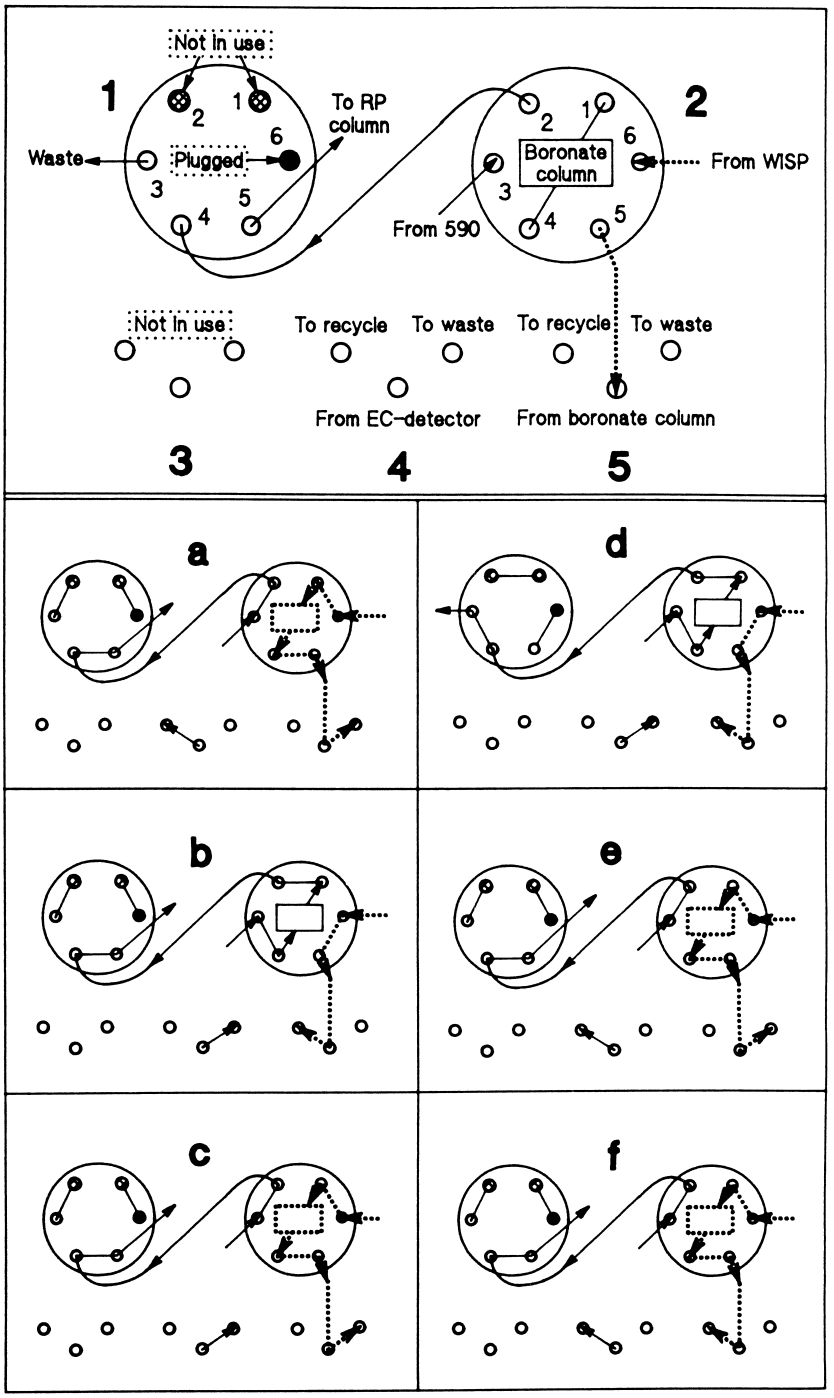


Fig. 7. HPLC chromatograms of a urine from a patient with malignant melanoma (from Ref. [141], with permission): (A) the urine was pretreated only on AG 50 W-X8, (B) the urine was pretreated only on alumina, (C) the urine after pretreatment with the combined extraction methods.

which became the standard method for many working groups worldwide for many years. This method works with only 1 ml plasma or 100 μl of urine or tissue extract. Besides 5-SCD, dopa and dopamine could be estimated. The methodical improvements consisted of: (i) the extractions being performed in substantial smaller vessels (1.5 ml, conic bottom), (ii) the amount of alumina being reduced to 50 mg, (iii) Tris buffer being used for adjustment of pH value to 8.6 and (iv) the elution being done with only 150 μl 0.4 *M* $HClO_4$. To remove some chromatographic disturbing peaks, the urines had to be precleaned by an acidic LLE with EtOAc. For plasma samples, there was no need for a deproteination. The absolute recovery for 5-SCD could be increased to 70%, the relative recovery was 93% for plasma and 99% for urine. A LOD of 0.16 n*M*/l was given. α-MD was used here as an I.S. After this extraction method very long chromatographic running times were necessary because of late interfering peaks. By an additional washing step during alumina extraction this time could be reduced to 45 min [162]. 5-*S*-Cysteinyl-α-MD was proposed as a more suitable I.S.

Scheibl et al. [178] could increase the absolute recovery of 5-SCD in plasma to 85% by the use of 0.2 *M* H_3PO_4 instead of $HClO_4$ for elution. Simultaneously, through this the stability of 5-SCD in the eluates was improved (I.S.: α-MD).

By these latter works it became clear that the


Not in use
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2
1
To RP column
Waste
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Boronate column
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From 590
4
5
Not in use
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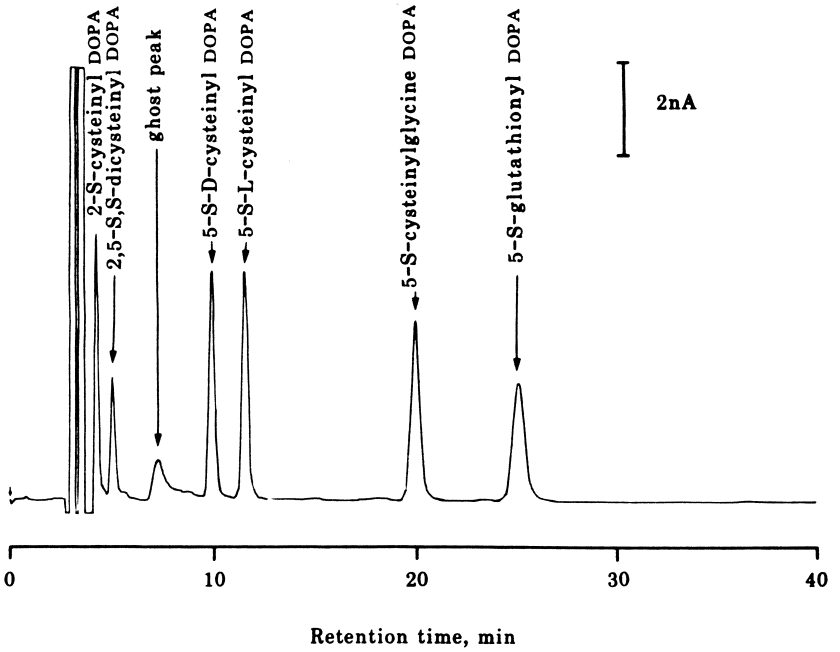

Fig. 9. Separation of a standard mixture of compounds of interest after injection into the boronate column and chromatographed after column switching on the reversed-phase column (from Ref. [163], with permission).

relatively low absolute recoveries for 5-SCD were not caused, as suspected in earlier works, by the alkaline pH value of 8.6 during sample pretreatment, but by unsuitable technical handling. This also corresponds with our own experience (J. Hartleb, unpublished results, 2001).

Finally, with regard to the alumina extraction technique, the following has to be noted: (i) it is easy to handle, with no additional costs and therefore commonly used, (ii) it is manually very extensive, and cannot be automated, therefore it is unsuitable for large sample series, (iii) the specimens are always processed without light protection, (iv) it is a batch-procedure, i.e., every sample has other pretreatment conditions and waiting periods up to injection. This is, particularly for sensitive substances, a substantial disadvantage, especially if plasma samples are processed.

4.1.2. Sample pretreatment with modified boronic acid affinity gels

In 1983, Kagedal and Pettersson reported on good experience with a sample pretreatment onto phenylboronate affinity gel of urine [158] and serum samples [179]. A quantitative adsorption of 5-SCD has already resulted with pH values $\geq$5.6, which indicates further interactions between the gel and the analyte. Also, here the elution is performed with acids (pH$\leq$3). The urines had to be pre-cleaned by a cation-exchange resin column. As an I.S. D-CD was

Fig. 8. Extraction and separation scheme with usage of an automated valve station (from Ref. [163], with permission). Both the high-pressure valves (1 and 2) and two of the three low-pressure valves (4 and 5) were used. Valve station from Waters, USA. Upper panel: plumbing for automated analysis of 5-SCD. Key to the lower panel: operation cycle of the automated determination of 5-SCD. Solid lines indicate mobile phase II (pH 3.0) and dotted lines indicate mobile phase I (pH 6.0). (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 5-SCD for 0.7 min. (c) Desorption is completed, and 5-SCD is chromatographed on the analytical column. (d) 5-SCD has now been detected. The high-pressure valve I is switched so that the boronate column can be washed for 3 min with mobile phase II. (e) Re-equilibration for 10 min of the boronate column is started. (f) The system is now ready for a new injection.

used. A relative recovery of 97% could be reached, the absolute one is indicated as quantitative.

In an improvement of this technique Hansson and co-workers [163,180] published an automated extraction method with a subsequent HPLC separation, realized by column switching. Using this method, specimens of 100 μl urine, but only urine, could be injected directly. Besides 5-SCD, 2-SCD, 6-SCD and 2,5-S,SCD also 5-*S*-cysteinyl-glycine-dopa and 5-*S*-glutathionyl-dopa could be analysed. The enrichment

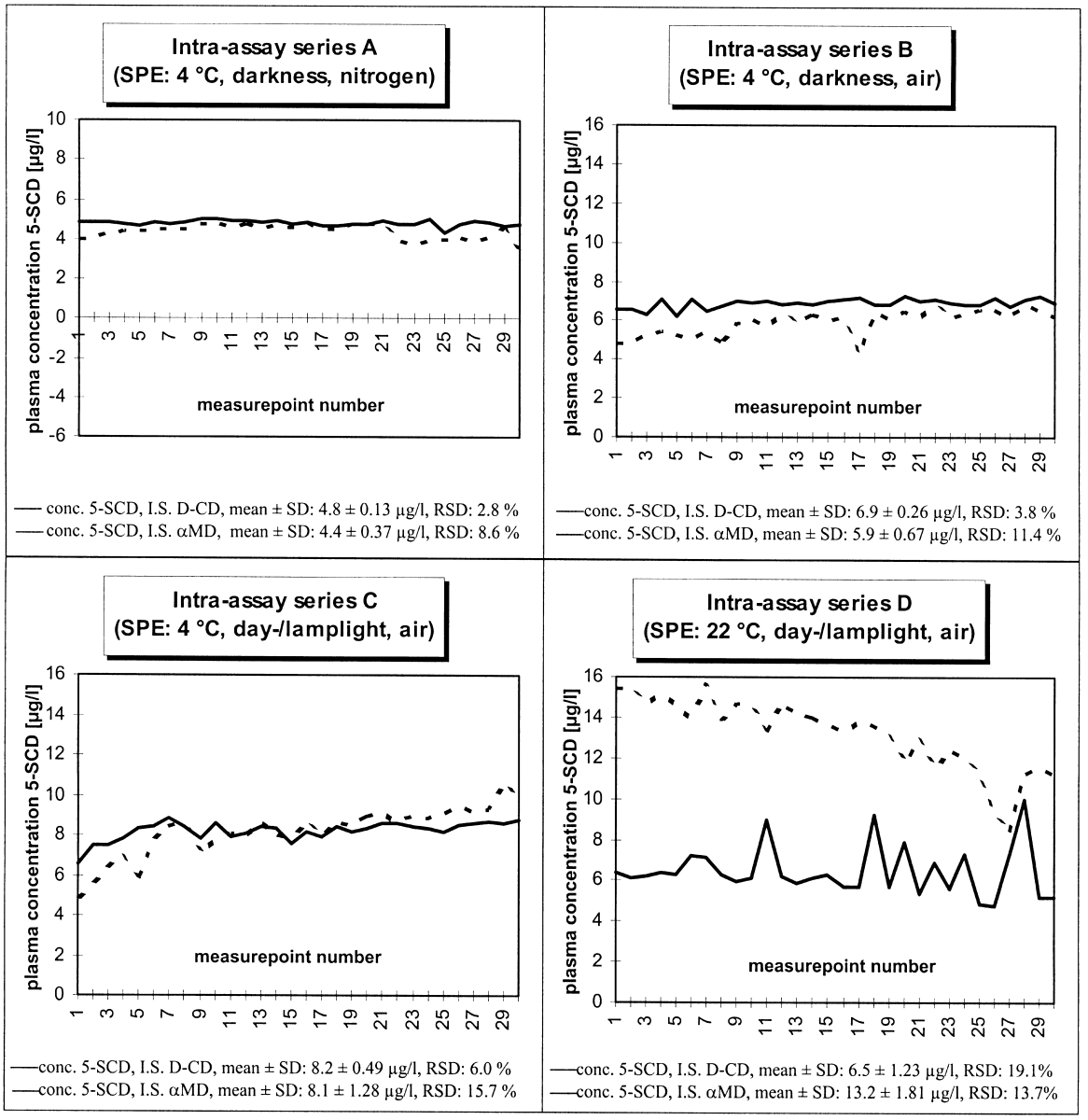

Fig. 10. The precision, correctness and course of 5-SCD concentrations in the intra-assay series A–D in plasma samples (a) after extraction processes with various conditions of temperature (b), light (c) and air–oxygen (d) and comparison of the suitability of two internal standards. (a) In each of the 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 (from Ref. [108], with permission).

material was acetylated boronic acid, bonded to silica (Pierce, Sweden). Details of this system and a chromatogram, resulting from this technique, are shown in Figs. 8 and 9.

For 5-SCD the absolute recovery after the boronate column was 92% and the relative recovery after the complete analysis was 93.5%. No I.S. was used, because D-CD and dopamine showed the same retention time. But, because of the nearly quantitative recoveries, there should be no need to use an I.S. [163]. Twenty-five urine samples could be analysed per day.

Summarizing this extraction technique, it can be stated: (i) it is a very suitable method, it shows nearly quantitative absolute and relative recoveries for urine samples and is thus better than alumina extraction, (ii) it offers the possibility of automation, i.e., lower manual effort, analysis of larger sample series, better protection against decomposition, more identical pretreatment processes for all samples, (iii) it requires additional HPLC equipment (second pump and column switching valves), (iv) up to this time it is not clear if this automated method is also suitable for analysis of the more meaningful plasma samples.

4.1.3. Sample pretreatment with solid-phase extraction

In 1988, Huang et al. introduced the SPE technique in the sample pretreatment of 5-SCD in urine [161]. For this purpose they used two commercial silica-based SPE materials, a strong cation-exchange (SCX) and a phenylboronic acid material (PBA), both from Analytichem (USA). In the first step the urines were loaded onto the acidic conditioned SCX cartridges and washed. Then the substances were eluted with 1 *M* K_2HPO_4 directly into the preconditioned PBA cartridges. After one more washing with water, 5-SCD was desorbed with 0.5 ml 0.1 *M* HCl (with 10 mg/l ascorbic acid). D-CD was used as an I.S. For 5-SCD a relative recovery of 96% and a LOD of 16 n*M*/l was found. No details were given about the absolute recovery.

The sample pretreatment by means of SPE has several advantages. The bonded-phase silica materials, in comparison to affinity gels, show a much better bed stability. This improves the reproducibility and saves time, because faster flow-rates of the solvents can be realized. Time-saving is possible by the simultaneous sample pretreatment of several specimens with the SPE vacuum station (at least 80 samples could be extracted per day). The cartridges can be used repeatedly [161]. Only urine samples were analyzed, no information was given about the suitability of this SPE technique for plasma samples.

In 1999 our working group published a new method for an automated determination of 5-SCD in plasma and urine, which overcame nearly all disadvantages of previous methods in sample pretreatment and analysis [108]. The sample pretreatment and immediate injection was carried out by means of an automatic SPE workstation ASPEC XLi (Gilson, France, and Abimed, Germany), which is the prerequisite of this method. As SPE material PBA was exclusively used, which yielded a sufficient selectivity. The very suitable D-CD served as I.S. Furthermore, this method is characterized by (i) cooling of all samples which were not in processing, (ii) extensive light protection during the sample pretreatment, (iii) use of nitrogen instead of air–oxygen and vacuum, respectively, during the SPE, (iv) minimal sample requirements (1 ml plasma, 50 μl urine), (v) large sample throughput (every 30 min a new sample can be analysed) and (vi) gaining almost identical analytical conditions for every sample as a result of automation. The relative recovery for 5-SCD in plasma and urine was 104%, the absolute recovery only 50%. The LOD in plasma was determined to be 0.44 n*M*/l. In this work, both pre-analytical and pretreatment parameters were investigated and their influence on the measured 5-SCD concentrations in plasma were shown (Fig. 10). In Fig. 11 a chromato-

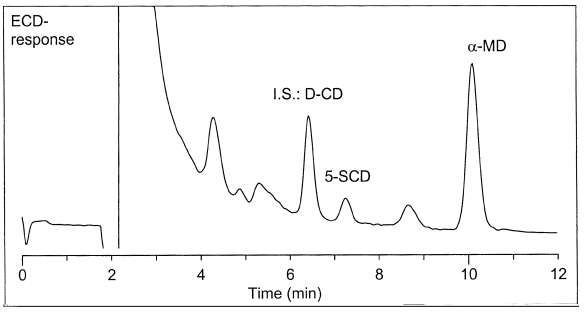

Fig. 11. Chromatogram of an extracted plasma sample of a healthy normal person (5-SCD plasma concentration 2.5 n*M*/l; the sample was analyzed according to Ref. [108] (from Ref. [154], with permission).

gram of a plasma sample of a healthy normal person, analyzed according to Ref. [108], is given [154].

4.2. Sample pretreatment of indole derivatives

4.2.1. Sample pretreatment for GC analysis

The majority of melanoma-related indole derivatives, with their polar functional groups, are quite unstable. They are slightly subject to an oxidation or polymerization [135], particularly the unconjugated substances. Therefore, GC-direct injection techniques, like headspace injections, are completely unsuitable. It is necessary to increase the thermal stability and volatility of these substances by derivatization.

For the investigations, urines were primarily used, cell extracts were rarely used [137]. Mostly LLE was used as extraction technology in early works with paper and thin-layer chromatographic analyses, as well as later with the GC. EtOAc [134,181] or diethylether [128,129,135] served as solvent. An acidic pH value was adjusted for the extraction of the indole carboxylic acids and a neutral pH value for the extraction of the neutral indoles. Sometimes a column-chromatographic pre-purification with DEAE-cellulose [128,129] or with poly-*N*-vinylpyrrolidon (PVP) [127] or a TLC preceded the LLE. Depending on the aim of the investigation, an enzymatic hydrolysis for decomposition of the conjugates was integrated into the pretreatment procedure (using of β-glucuronidase, arylsulphatase or *Helix pomatia* juice).

The derivatization before the injection into the GC was performed mainly with PFPA (for hydroxyl groups) and/or HFIP (for carboxyl groups) [128,129,135,137], but also a trimethylsilylation with BSTFA [127,134], *tert*.-butyldimethylchlorosilane-imidazole [127] or methyl-8 with Tri-Sil/TBT (Pierce, USA) [181] was carried out.

4.2.2. Sample pretreatment for HPLC analysis

The number of publications on the analysis of melanoma-related indole derivatives by means of HPLC is quite low. The sample pretreatment was deduced from GC methods.

Pavel et al. first published a complete HPLC methodology for urine analysis of both indole and indole carboxylic acid derivatives [75]. The sample pretreatment was realized by means of LLE. The urines, without or after enzymatic hydrolysis, were mixed with the I.S. (5-methoxyindole-2-carboxylic acid) and then saturated with NaCl. The LLE was performed twice with 4 ml diethylether at pH 6.2. After evaporation of the pooled extracts with nitrogen, the samples were redissolved in the HPLC eluent and 20-μl aliquots were injected into the HPLC. Fig. 12 shows chromatograms of an extracted urine of a normal person, without and after enzymatic hydrolysis of glucuronide and sulphate conjugates [75].

Unfortunately, no details of the method characterization were given. This would be interesting, since the extraction was carried out at pH 6.2, which is nearly neutral. Under these conditions different recoveries for indoles and indole carboxylic acids would be expected.

By means of LLE (EtOAc) the indole carboxylic acids could also be extracted from serum [104,105,120]. The pH value at the extraction had to be explicitly in the acidic range to reach a sufficient recovery (5H6MI2C, 52/66%; 6H5MI2C, 71/74%; 5,6DHI2C, 20%). In contrast with neutral pH values, recoveries of only 20% were found. In the latter work no I.S. was used, instead measurements were only corrected in principle for the determined recovery. For the extraction of the sulphate and glucuronide conjugates of 5H6MI2C and 6H5MI2C from urine, LLE at pH 1 was used [136].

In 1988, Wakamatsu et al. showed for urines samples [101] that LLE pretreatment is not required in the case of subsequent HPLC separation. After centrifugation, 10 μl of the urines were injected directly, as later practiced also in Ref. [95]; thus, the main components 5H6MI2C and 6H5MI2C could be analyzed. This direct injection technique could be used also for tissue and cell homogenates after centrifugation [102,104]. No I.S. was applied in these methods.

Determination of 5,6DHI2C in urine samples was also possible after enrichment of this substance onto alumina [118,147].

Until now, no systematic investigations on the pre-analytical influences and the stability of the samples and substances during sample pretreatment, exist. After the results of such studies for 5-SCD,

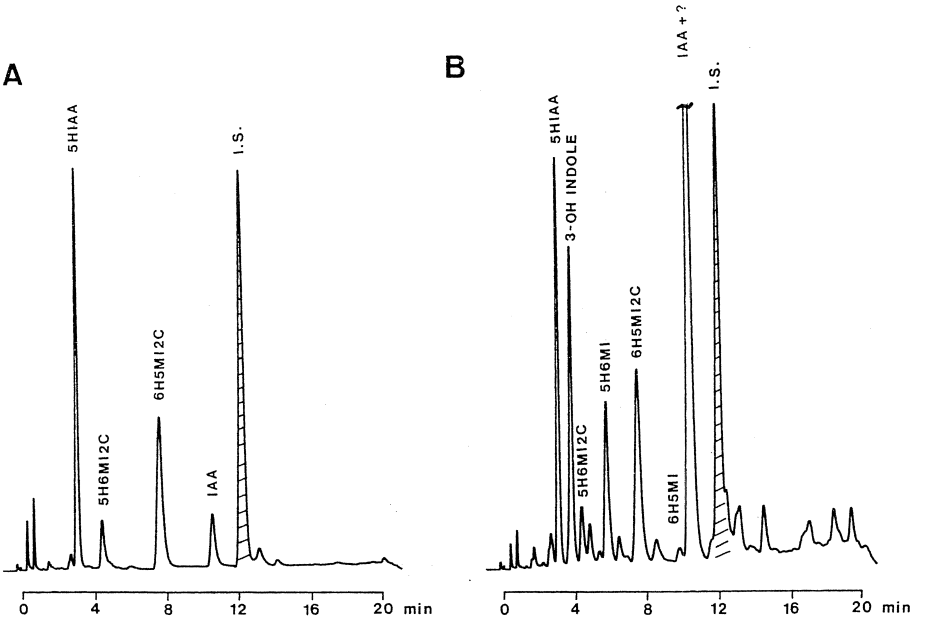

Fig. 12. Chromatograms of an extracted urine of a normal person, (A) without and (B) after enzymatic hydrolysis of glucurono and sulpho conjugates (IAA, indolyl-3-acetic acid; 3-OH-indole, 3-hydroxyindole) (from Ref [75], with permission).

this would be also of interest for the indole derivatives, especially in plasma samples.

5. Internal standards

5.1. Internal standards for cysteine derivatives

For the analysis of 5-SCD, the use of an I.S. was approved by most authors. Some more or less suitable I.S. can be selected. In early works, isoproterenol was often used [182]. But this I.S. was not very suitable, as already shown by the different recoveries (5-SCD, 31%; isoproterenol, 81% [87]). Another substance is α-MD, used in many studies and often on alumina extraction [144,178,183]. Although the absolute recoveries for 5-SCD and α-MD after alumina extraction were nearly identical, this substance is not suitable to correct the differences in the complete analytical process. Wakamatsu and Ito also gave this assessment [162], although they introduced this I.S. in 1984. We found that irregular results were obtained by the calculation of 5-SCD relative to α-MD [108]. With α-MD, the sample pretreatment onto alumina is probably well corrected; however, this cannot be stated for the SPE onto PBA. Especially, the light and oxidation sensitivity of 5-SCD is reflected very inadequately by α-MD. Wakamatsu and Ito suggested 5-*S*-cysteinyl-α-MD as a more suitable I.S., which, from a chemical view, really should be more suitable [162].

Already in 1983, Kagedal and Pettersson [158] suggested as I.S. a stereoisomer of 5-SCD, D-CD. This can be simply prepared and easily separated from 5-SCD under normal HPLC conditions. Therefore, but primarily because of its structure, D-CD should be a nearly ''ideal'' I.S. This could be confirmed in some investigations [108,161], but even D-CD is not ''ideal'' in every case (see Fig. 5). In conclusion, for HPLC it is the I.S. of choice.

The isotope-labelled original cysteinyl compounds are of course other ''ideal'' internal standards. But these substances require additional equipment, namely a radiometric or a MS detector. A tritium-labelled 5-SCD was used only once by Kagedal and Pettersson [141].

5.2. Internal standards for indole derivatives

For the early analyses of melanoma-related indole derivatives with GC–MS, almost deuterated substance analogues came to be used as I.S. As expected, these substances proved very suitable [130,135,137].

For the correction of analyses by means of HPLC after LLE, at first 5-methoxyindole-2-carboxylic acid (5MI2C) was used [75], but in this work no explicit details on the suitability of this substance as I.S. were given. In later HPLC measurements it was completely renounced for use as an I.S. [101, 105,120]. In the case of direct injection of urine samples such a practice is understandable, but in the case of LLE of plasma samples this is surprising. One possible reason for this may be the insufficient suitability of 5MI2C, because this substance has no hydroxyl groups at the ring, as do 5H6MI2C and 6H5MI2C.

On no account was an I.S. used for correction of the enzymatic hydrolysis during the sample pretreatment.

6. Chromatographic analysis

6.1. Analysis of cysteine derivatives

6.1.1. Early techniques

The first measurements for the determination of 5-SCD in tissues and cell samples [80], after sample pretreatment onto alumina, were performed by fluorometry. The starting point was methods for the determination of catecholamines [176,184], for which the compounds were oxidized with $K_3[Fe(CN)_6]$ to yield fluorescent compounds. After improvements in the methodology by oxidation with $NaIO_4$ [177] and still further with iodine [81], it was possible to determine 5-SCD in urines of normal persons. This method failed, however, for measurements of 5-SCD in plasma samples. Finally, it must be said, that these methods showed only an insufficient sensitivity and specificity.

In the analysis of the melanoma-related cysteine derivatives, GC–MS plays only a minor role [185]. After pretreatment onto alumina or ion-exchange columns, the derivatization of the samples was performed with PFPA. The separations were carried out on glass columns containing 3% OV-17 on Chromosorb W. The ionization was performed with an EI energy of 70 eV. By this technique, different cysteinyl-dopa-isomers and some of their *O*-methyl derivatives in urine of melanoma patients could be separated.

Other early analysis techniques, e.g., ion-exchange column chromatography with effluent monitoring using the stable free radical α,α-di-phenyl-β-picrylhydrazyl as a colorimetric reagent [167], proved to be meaningless.

6.1.2. HPLC

With the technical development and wide distribution of the HPLC at the end of the 1970s, this technique was used preferentially for the determination of the melanoma-related cysteine derivatives. From the beginning, the separation of cysteine compounds was a not too difficult task for the analysts.

The first HPLC separation of 5-SCD was described by Hansson et al. in 1978 [87]. After pretreatment onto alumina, serum samples were analysed. As a separating column, a 200×4-mm Nucleosil RP18 with 5 μm particle size was used. The eluent consisted of 2.9 g/l H_3PO_4 in water with 0.5% MeOH. The retention time of 5-SCD was 8.4 min; as detector, an ECD with a carbon paste electrode was used (+0.75 V versus Ag/AgCl).

Later Hansson et al. also achieved the separation of the other *S*-cysteinyl compounds in serum and urine [186]. In this work the authors carried out extensive systematic examinations on many factors influencing the separation, e.g., different column materials, ionic strength and pH values of the eluent and use of different ion-pair reagents in the eluents. The best separation results here were achieved using the ion-pair reagent methanesulphonic acid.

Generally, for HPLC analyses, usual RP materials were used, mostly C_{18}, seldom C_8 materials [141,158,179] with particle sizes of almost 5 μm in columns of 100–250 mm length and 4.0–4.6 mm I.D. The eluents consisted of 30–150 m*M* buffers (phosphate, formate [163] or citrate [159]) with 0.1–0.2 m*M*/l EDTA and often with ion-pair reagents (mostly 40–100 m*M* of methanesulphonic acid, but also with other alkylsulphonates) with pH values of

approximately 2–3. Organic components, like methanol, were used only with portions round 1%. The analyses was usually carried out isocratically.

As detectors, electrochemical detectors (amperometrically) were exclusively used: initially with carbon paste electrodes, later with glassy carbon electrodes. The applied potentials were between +0.45 and +0.75 V (versus Ag/AgCl reference electrode). The addition of EDTA to the eluent is substantial to improve the stability and suitability of the ECD in daily routine work [141].

6.2. Analysis of indole derivatives

The existence of indole derivatives in the urine of patients with melanoma was suspected in older studies using PC methods. Substance spots after PC separation showed a specific colour reaction (Thormählen reaction [123]). These ''Thormählen positive substances'', however, could not be identified.

The first meaningful investigations of the melanoma-related indole derivatives at the end of the 1970s, resulted mainly from the work of Pavel in some working groups. For the identification and elucidation of structures, GC–MS was almost exclusively applied. With the increasing use of HPLC in biochemical analysis, and considering the information about the structure and metabolism of melanoma-related indole derivatives which had been uncovered, soon GC was completely replaced by HPLC.

6.2.1. GC

Most separations were performed on fused-silica capillary columns (length 15–25 m, I.D. 0.25 mm), coated with phases like SE 54, SE 30 or CP-Sil 5 [127–129,135,137]. Duthel and Vallon [181] used a 30 m×0.75 mm wide-bore borosilicate glass column, coated with SPB 1 for the separation. Because of the high separating performance of the used capillary columns, the separation of the substances was not a critical point.

Since the primary interest was the elucidation of the structures, mainly MS detectors were used (TIC and SIM mode). These detectors have advantages in sensitivity compared with a normal FID, furthermore deuterated substance analogues could be used for identification and quantification. The ionization was normally carried out with EI (70 eV). Pavel et al. showed that with chemical ionization (methane; EI 180 eV) a safe fragmentation and determination is also possible. Fig. 13 shows mass fragmentograms, obtained with the latter technique, from a derivatized (PFPA and HFIP) extract of a melanotic urine, to which deuterated I.S. were added [135].

6.2.2. HPLC

The HPLC separation of the melanoma-related indole derivatives caused few difficulties. Normal RP18 materials (RP8 in Ref. [75]) with particle sizes of 5–7 μm in columns of 100–250 mm length and I.D. of 4.6–5.0 mm were used.

The eluents consisted of buffers (e.g., 50 m*M*/l of sodium acetate or 100 m*M*/l of potassium phosphate each with 1 m*M*/l EDTA) with pH values of 2.1–4.3. As organic component, methanol was used in portions of 12–20%. The analyses were usually carried out isocratically. Two different detectors came into use; the first one being the fluorescence detector. The indole derivatives show a very sensitive and selective native fluorescence. For excitation/emission wavelengths, the following values were applied: 305/360 nm for all indole derivatives [75], 315/390 nm for the indole carboxylic acids [101] and 315/420 nm for the sulphate conjugates of the indole carboxylic acids [102].

Electrochemical detectors (amperometrically) with glassy carbon electrodes became the second type of detector. The ECD has, at the relatively low potentials which can be used for the detection of the indole derivatives, a very high selectivity at very high sensitivity. So potentials of, e.g., +0.75 V [102], +0.40 V [104,105] and +0.60 V [95,120] (versus Ag/AgCl reference electrode) could be applied. With the ECD, of course, only such indole derivatives can be detected which have free hydroxyl groups bound to the indole ring.

7. Special clinical and biomedical applications

7.1. 5-SCD and amelanotic melanomas

The bibliographical references on the suitability of 5-SCD for the detection of amelanotic melanomas

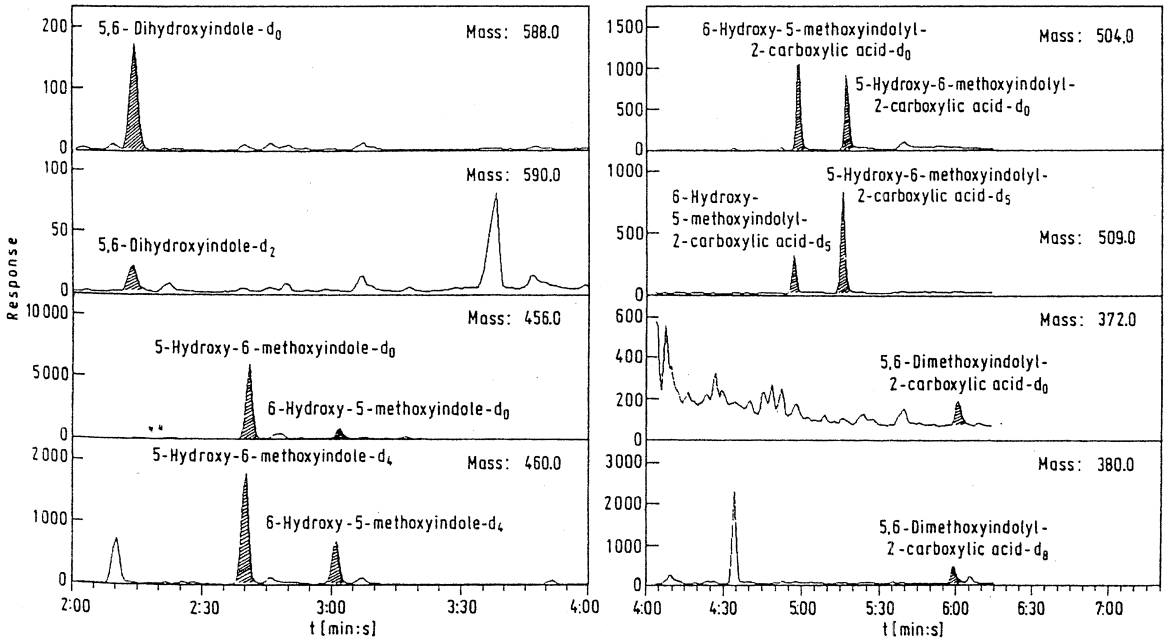

Fig. 13. Mass fragmentograms obtained with chemical ionization (methane) technique from a derivatized (PEPA and HFIP) extract of a melanotic urine, to which deuterated I.S. were added (from Ref. [135]), with permission); d_x, *x*-fold deuterated.

and metastases in amelanotic melanomas, respectively, are contradictory. One reason for this is the rare appearance of this form of melanoma. But also analytical difficulties, such as the lack of sensitivity, probably might play a role.

Mojamdar et al. [99] did not find any 5-SCD in amelanotic melanomas or other non-pigmented tissues, and no tyrosinase activity in amelanotic melanoma cell lines [187]. Hu et al. [103] injected cells of an amelanotic melanoma in mice and could not detect 5-SCD in the plasma in the period following. Contrary to this, Morishima et al. found 5-SCD also in amelanotic melanoma cells [188]. The 5-SCD concentration in these tissues, however, is lower than in melanotic melanoma cells, but comparable with the one in pigmented nevi. This was also confirmed by Hara et al. [92], who found measurable 5-SCD values in primary and metastatic amelanotic melanomas.

Peterson et al. [106] found increased 5-SCD serum values only in two of five patients with amelanotic melanomas in stage IV. Horikoshi et al. observed normal 5-SCD values in urine and serum in two amelanotic melanomas without metastases [107]. However, the values of these patients increased after the appearance of metastases. Wimmer et al. [119] found normal 5-SCD serum values in two patients with primary amelanotic melanomas. After development of metastases, the 5-SCD concentration increased in one case and remained normal in the other case. In an extensive study by Banfalvi et al., in all seven patients with primary amelanotic melanomas (three patients in stage I+II, three patients in stage III and one patient in stage IV with lung metastases) no increased 5-SCD serum levels were observed [121]. Also, Hirai et al. [41] found in all three patients with amelanotic metastases normal 5-SCD values.

In summary, a distinct 5-SCD concentration seems to exist also in amelanotic tissue or cell samples, which however does not exceed the one in congenital or dysplastic nevi. But also in such tissues, only in 20 of 35 cases could increased 5-SCD contents be detected [155]. No 5-SCD was found in any of 35 uninvolved skin samples. These low 5-SCD concentrations in the cells are not necessarily reflected in increased 5-SCD urine values, which are more likely in plasma. Whether or not increased plasma 5-SCD

was found after metastasis of primary amelanotic tumours presumably depends on whether the metastases were amelanotic or melanotic (what often is not indicated). It could also depend on the place of the metastases formation or, as with melanotic melanomas, of other unfortunately still unknown factors.

7.2. 5-SCD and 6H5MI2C and melanomas in children

Malignant melanoma in children and adolescents is a very rare disease. The incidence in prepubertal children is approximately 0.3–0.4% of all melanomas [189,190]. In the second decade of life, the incidence increases 2–7-fold [191–193]. The situation is complicated by the difficulties in identifying and in differentiating the melanoma tumours from other common benign skin diseases (e.g., from ''Spitz nevus'') [194]. So the problem of a histological overdiagnosis is considerable [193].

In 1996, an overview was published on all cases of malignant melanoma which occurred in Scotland in the years 1979–1991 [193]. From the recognized 4700 cases, only in 50 cases were the patients 18 years or younger (1.04% of all melanomas). In nine of these 50 cases metastases were found. From those nine, eight patients died with a maximum survival of 4 years.

From clinical observations it has been known for a long time that there is an increased risk of melanoma in patients (usually children) with giant congenital pigmented nevi (GPN). However, this risk has not yet been quantified. Also, the definition of a GPN differs among authors.

Swerdlow et al. recently published in a cohort study [195] data from 265 patients with congenital nevi, most of them (94%) were 19 years old or younger. Thirty-three of these patients (12%) had their largest nevus covering 5% or more of the body surface (26% covering 1–4%, and 62% covering $<$1% of the body surface). A strong correlation was found between the size and the number of nevi. Two of the six deaths which occurred in the cohort could be followed back to development of a melanoma. In both deaths the largest nevus covered more than 25% of the body surface. Because of these data, there is a 1000-fold increased risk of melanoma mortality in patients with nevi $>$20% of the body area, but the absolute risk is not as great as clinically suspected. A similar result was found in Ref. [193]. There was no case of a melanoma observed in paediatric patients with GPN.

Hanawa et al. [196] published investigations of 5-SCD in serum (after alumina extraction) from children with GPN ($\geq$2% of the body surface) but without malignant melanoma in comparison to paediatric patients with smaller congenital nevi (CN) and to patients with non-melanocytic benign skin tumours. Abnormally high 5-SCD concentrations were observed in 38% of the 21 patients with GPN, in 23% of the 22 patients with CN but also in 7% of the 26 patients (two cases) with non-melanocytic diseases. For the 5-SCD concentration a positive correlation (for children $<$5 years and $>$5 years) with the size of the GPN was found and a negative correlation with the age of the patients in all three patient groups.

Measurements of 5-SCD (after automated extraction with boronic acid affinity gels) and 6H5MI2C (after LLE) in random spot urines of 136 healthy children (5–15 years) were recently published by Meyerhöffer et al. [142]. The concentrations of 5-SCD (0.30–0.44 $\mu M/l$) and 6H5MI2C (0.18–0.32 $\mu M/l$) in all subgroups (age gradation every 2 years) were roughly the same, while the concentration of creatinine, as expected, increased with age. Thus, for the values of 5-SCD and 6H5MI2C related to creatinine, an age-related decrease of the means was yielded (5-SCD: 60.4–28.0 $\mu M/M$ creatinine; 6H5MI2C: 42.8–26.1 $\mu M/M$ creatinine; in the subgroups from 5 to 15 years). This would be a good correlation with the observed age-dependence of the 5-SCD serum concentration in Ref. [196], but for young adults (average 23 years) a distinct increase of the values was found (5-SCD: 49.0 $\mu M/M$ creatinine, 6H5MI2C: 33.4 $\mu M/M$ creatinine), which is difficult to understand.

Because of the measured sensitivities of the discussed substances in the different matrices, as well as the natural problems with the urine sample collection in children (whether random spot or 24 h collecting urine), for children an analysis of plasma samples should be preferred.

8. Summary and conclusions

For investigations of the melanoma-related cysteine and indole derivatives, GC and HPLC were primarily applied. GC–MS was used mainly in earlier years, in the initial phase of the biomedical research in this fields. That was necessary, since the elucidation of structures and identification of substances were in the foreground. However, for a long time the analyses were carried out exclusively by HPLC. This was because of the lower effort necessary for sample pretreatment, including the no longer necessary derivatization procedures. Until now, for these substance groups the use of a HPLC–MS coupling has not been described. And there also has not been any application of modern capillary electromigration techniques yet.

In the group of the melanoma-related cysteine derivatives, 5-SCD is obviously the most important substance. Other isomers or intermediate products do not have any meaning as marker substances. Either they are too reactive, are found in concentrations too low or they do not allow any other statements to be made, which cannot be made by 5-SCD. In the group of the melanoma-related indole derivatives, 5H6MI2C and 6H5MI2C seem to be best suitable for analysis and as marker substances. So only the main components 5-SCD and 6H5MI2C were investigated in most studies.

Preceding the chromatographic analysis of both analytes a sample pretreatment is needed. There are three techniques for the pretreatment of 5-SCD with different specifications. The clean-up onto alumina can be carried out simply and is also common, but it cannot be automated and it also has other disadvantages. The sample pretreatment onto boronic acid affinity gels yields near-quantitative recoveries and enables automation by means of column switching. However, this technique has only been tested with urine samples until now. Finally, the pretreatment can be carried out with the SPE technique using SCX+PBA or only PBA cartridges. This technique is suitable for urine and plasma samples. A full automation can be realized and in this case it is the best methodology for the investigation of plasma samples. For the sample pretreatment of the indole derivatives (usually 6H5MI2C) the LLE is primarily used, both for urine and for plasma samples. Within the last few years urine samples were also directly injected into the HPLC system. This is an elegant analysis technique, but is applicable only for the main components.

The application of a suitable I.S. is important for the correctness of the measured values. For 5-SCD, several possible substances are available for use, but not all of them are really suitable. For the indole derivatives the estimations were usually done without the aid of an I.S.

The chromatographic analyses with HPLC or GC/GC–MS could be realized in most cases without major problems. Also, in the past, the performance of capillary GC and HPLC was usually sufficient to separate the substances of interest. For GC–MS analyses, the separations were performed with normal fused-silica capillary columns, coated with relatively non-polar liquid phases after derivatization with PFPA, HFIP or BSTFA. MS detection was mainly carried out with EI ionisation. For HPLC, standard RP18 separating columns and the usual compositions of eluents (partly with ion-pair reagents) were used. As detectors, both ECD and FD showed a sufficient sensitivity and selectivity.

Based on their chemical structures and confirmed in examinations, all substances in these two groups are very sensitive to light and oxidation. These properties must be taken into account in the complete analysis procedure, from blood withdrawal or urine collection, throughout the pretreatment technique used, up to injection into the chromatographic system. If these decisive parameters are ignored, false low values will result. Because of the approximately 100 times lower concentrations of 5-SCD and 6H5MI2C in plasma in comparison to urine, this is particularly essential when measuring plasma samples. Unfortunately, these considerations are only partly realized in the analytical methods. Systematic examinations of these sensitivities exist for 5-SCD in plasma and urine, as well as partially for 6H5MI2C in urine. So, for a critical discussion of the analytical methods and still more for the interpretation of the obtained results, the detailed analytical procedures must be considered.

Serological markers for malignant melanoma are advantageous for several reasons. They make it

possible to obtain economical findings about the existence or the stage of this disease. Regular control measurements are possible and these are non-invasive for the patients. The following questions are important for the suitability of substances as markers of malignant melanoma: are the markers suitable for early diagnosis? Are they suitable for detection of progression of the disease? Are they suitable for monitoring the course of therapies? And can they give statements about the prognosis for the patient? The ''ideal'' marker, which can safely answer all questions, does not exist, either by cysteine or indole derivatives, or by other marker substances discussed in this field.

Two principle cases have to be distinguished from malignant melanoma, primary melanomas without metastases (disease stages I and II) and metastatic melanomas (stages III and IV). For all marker substances tested until now, also for 5-SCD and 6H5MI2C, the sensitivity of the measured results has depended strongly on the melanoma stage, i.e., pathological values are obtained increasingly only in the metastatic stages.

5-SCD in plasma, if analyzed using a suitable technique, is the best marker of malignant melanoma apart from S100β. It shows an excellent specificity and also an adequate sensitivity in melanoma stages III and IV. Regarding the detection of primary melanomas, 5-SCD is perhaps slightly better than other markers, but here the sensitivity is altogether low. For urine samples the sensitivity of 5-SCD is generally lower than for plasma samples, and this could not be increased significantly by using sophisticated statistical methods. 5-SCD is also very suitable for therapy monitoring and for the detection of a metastasis in an early stage. Unfortunately, in comparison with, e.g., S100β, the oxidative sensitivity of 5-SCD in plasma retards its routinely use. The very good marker qualities of 5-SCD in plasma can only then be obtained, if the samples have been obtained and analysed under optimal conditions. This effort presumably was difficult to realize by many working groups, because there have only been a few studies of 5-SCD in plasma.

6H5MI2C and other indole derivatives have been investigated far less than 5-SCD. 6H5MI2C correlates less clearly with the different stages of melanoma and usually shows pathological values only in the final stages. Therefore, as a marker for malignant melanoma, 6H5MI2C is less suitable, it reflects more the general status of the pigmentation of the skin. However, a detailed examination of the pre-analytics and the stability of 6H5MI2C in plasma at sample pretreatment and analysis has not been carried out until now. Here, one can expect similar results as for 5-SCD. Moreover, an investigation of the different indole derivatives in plasma and urine after enzymatic hydrolysis has not been carried out on large patient groups. From such studies, some new findings could be possibly won, which could lead to a new evaluation of the significance of melanoma-related indole derivatives as markers of malignant melanoma.

For future examinations the following requirements seem to be important to increase the sensitivity of the markers of malignant melanoma, and with that to increase the benefit for the patients.

Instead of the analysis of a single marker, the simultaneous measurement of several different markers in a given patient sample should be performed. Apart from very few exceptions, there are no studies which are related to single patients. Usually the sensitivities of each of the measured markers was calculated relating to a patient group. However, the response of different patients to different markers varies. So, an improvement of the diagnostic finding can be expected, if all of the pathological values are considered, independent of their origin. Such a multimarker analysis should not be restricted only to 5-SCD and 6H5MI2C, but also extended to markers with other biochemical mechanisms.

Not only one measurement should be carried out per patient. It would be more meaningful to observe the patients in the follow-up with laboratory diagnostics. Considering the relatively low costs of the laboratory measurements of the markers, compared to the costs for conventional staging, therapy and especially for surgical treatments, this should be feasible.

Clinical, histological and/or biological classification of malignant melanomas and their metastasis do not seem to be sufficient, which would explain the lack of sensitivity of all markers tested. This could be an important approach to clarifying inexplicable differences in the response of the patient groups until now. For further studies, patient groups which are

more homogeneous should be examined. This also concerns the definition of patient stages. It would be more meaningful, if the current clinical picture of the patients would be taken more into account.

9. Definition list

5-SCD	5-*S*-L-cysteinyl-L-dopa
2-SCD	2-*S*-L-cysteinyl-L-dopa
6-SCD	6-*S*-L-cysteinyl-L-dopa
2,5-S,SCD	2,5-S,*S*-di-L-cysteinyl-L-dopa
D-CD	5-*S*-D-cysteinyl-L-dopa
Dopa	L-3,4-dihydroxyphenylalanine
Dopac	3,4-dihydroxyphenylacetic acid
5,6DHI	5,6-dihydroxy-indole
5H6MI	5-hydroxy-6-methoxy-indole
6H5MI	6-hydroxy-5-methoxy-indole
5,6DMI	5,6-dimethoxy-indole
5,6DHI2C	5,6-dihydroxy-indole-2-carboxylic acid
5H6MI2C	5-hydroxy-6-methoxy-indole-2-carboxylic acid
6H5MI2C	6-hydroxy-5-methoxy-indole-2-carboxylic acid
5,6DMI2C	5,6-dimethoxy-indole-2-carboxylic acid
5-HIAA	5-hydroxy-indole-3-acetic acid
α-MD	α-methyl-dopa, 2-methyl-3-(3,4-dihydroxyphenyl)-L-alanine
COMT	catechol-*O*-methyl-transferase
EGTA	[ethylene-bis(oxyethylenenitrilo)]tetraacetic acid
EDTA	ethylene-diamine-tetraacetic acid disodium salt
DTPA	diethylene-triamine-pentaacetic acid
EtOAc	acetic acid ethyl ester
GSH	reduced glutathion
Tris	tris(hydroxymethyl)aminomethane
SPE	solid-phase extraction
LLE	liquid–liquid extraction
PBA	phenylboronic acid
SCX	strong cationic exchange
TLC	thin-layer chromatography
PC	paper chromatography
HPLC	high-performance liquid chromatography
RP	reversed-phase
ECD	electrochemical detector
FD	fluorescence detection (excitation/emission wavelength)
GC	gas chromatography
MS	mass spectrometry
TIC	total ionic current
SIM	single ion monitoring
EI	electron-impact ionization
FID	flame ionization detector
PFPA	pentafluoropropionic acid anhydride
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
BSTFA	bis(trimethyl-silyl)trifluoroacetamide
I.D.	internal diameter
C.V.	coefficient of variation
SD	standard deviation
I.S.	internal standard
M.W.	molecular mass
LOD	limit of detection

Acknowledgements

The authors wish to thank Dr. S. Keller for critical reading of the manuscript and Mrs. Leonie Lienau for assistance in writing this review.

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