Talanta 84 (2011) 280–286

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Pre-study and in-study validation of a SPE-LC–MS–MS method for the determination of 5-S-cysteinyldopa, a melanoma biomarker, in human plasma

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ARTICLE INFO

Article history:
Received 13 July 2010
Received in revised form
20 December 2010
Accepted 26 December 2010
Available online 13 January 2011

Keywords:
5-S-cysteinyldopa
Method validation
LC–MS–MS
Plasma
Melanoma biomarker

ABSTRACT

The incidence of malignant melanona has increased over the past decades, particularly in Caucasian population. This disease presents defavourable prognosis in terms of survey, especially when detection occurs at the metastatic phase. Reliable analytical methods for biomarker determination are thus an interesting tool in pathology detection and follow-up. In this context, a method using SPE-LC–ESI–MS–MS for the determination of 5-S-cysteinyldopa (5-SCD) in human plasma was optimized. The presence of matrix effect was investigated in details while 5-SCD stability was studied according to FDA requirements for the validation of bioanalytical methods. Pre-study and in-study validations of the entire method were then successfully performed by applying the approach based on total measurement error and accuracy profiles over a concentration ranges from 1.6 to 200 ng/ml. Good results with respect to accuracy, trueness and precision were obtained. The maximum risk of observing future measurements falling outside the acceptance limits during routine analysis was also estimated.

1. Introduction

The term melanoma indicates a malign tumor developed at the expense of melanocytes. It affects principally the skin, rarely the nails or the mucous membrane. Melanoma represents less than 10% of skin cancers but is responsible of 80% mortality due to this type of cancer. Prognosis in terms of survey is in relation with cancer stage at the first clinical presentation and is particularly unfavourable for metastatic melanoma. For the 30 last years, incidence of melanoma has been growing, in the light colour skin population in majority but seems to have reached a plate in some parts of the world due to prevention campaigns [1–3].

Early detection of melanoma is thus of utmost importance essentially due to the limited choice of therapeutics. Tumor excision remains now the treatment of choice for early stage melanoma. If needed, adjuvant chemotherapy or biochimiotherapy (i.e. interferon-α and interleukin-2) can also be prescribed [1,4–6]. In addition to the histologic exam of biopsy, medical imagery techniques are principally used as diagnostic tools. The most popular methods are echography, MRI, tomography and scintigraphy. Nevertheless, micro-metastasis may escape to their detection.

Interest in finding new melanoma markers led to the evaluation of different molecules such as S100B protein, Ki-67, HMB45, osteopontin, MIA factor, etc. [4,7–23]. These markers are often significant for advanced stages of malignant melanoma but are sensitiveless in the early stages (except HMB45) [14]. In addition, specificity of these markers is not always completely ensured. Among blood markers, 5-SCD (Fig. 1), an intermediate in the metabolic pathway of pheomelanin synthesis, seems to constitute one of the best markers of melanoma detection and progression in the metastasis development (stages III and IV) [4,10,24–27]. 5-SCD sensitizes DNA to UV oxidative damages, indicating its implication as risk factor in melanoma induction by ultraviolet radiations [28]. But, in the same time, it protects melanocytes from membraneous damages (permeability) induced by the same kind of radiations, UVA particularly [28].

The elevation of 5-SCD plasma concentrations would precede clinical appearence of metastasis and their detection by classical imagery methods, establishing a real prognostical interest for

Abbreviations: 5-SCD, 5-S-cysteinyldopa; AAPS, American Association of Pharmaceutical Scientists; ESI, electrospray ionization; DA, food and drug administration; LC, liquid chromatography; LLOQ, lower limit of quantification; MeD, methyldopa; MRM, multiple reaction monitoring; MS, mass spectrometry; SPE, solid phase extraction; ULOQ, upper limit of quantification.

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doi:10.1016/j.talanta.2010.12.047

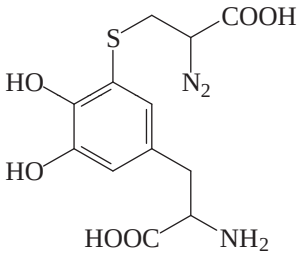

Fig. 1. 5-SCD strucutre.

cysdopa. 5-SCD would be a useful marker for the follow-up of patients treated by immuno- or chemotherapy because it would allow distinguishing responsive from non-responsive patients. Indeed, a decrease of the plasma concentration of this marker was observed within 3–5 weeks after a successful treatment (resection or therapy). 5-SCD would thus reflect the sickness stage with a short latency of response. The 5-SCD rates appeared to be uninfluenced by other tumors or skin pathologies ensuring a high specificity for this marker. Nevertheless, exposition to UV radiation influences 5-SCD levels. Wolber et al. would have recently established that cysdopa concentration can be 4 times higher for the skins exposed in a repetitive way to UVB radiations for 2 weeks [29].

Environmental and physical factors, as well as sometimes unappropriated analytical conditions with respect to 5-SCD sensitivity towards oxidation, led to doubtful determination of normal values and sensitivity of this biomarker. Nevertheless, plasma concentrations of 1.6 and 3.2 ng/ml seem to be generally accepted as reference values corresponding to the maximum of normal rate and the pathological threshold, respectively. It is worth noting that plasma concentrations of 5-SCD should be more reliable than urinary ones even if they are approximately 100 times lower. Indeed, metabolic processes such as oxidation, methoxidation or conjugaison influence the urinary content in 5-SCD.

Until now, 5-SCD determination was performed by chromatographic methods associated with electrochemical detection [4,26,27,30]. We previously developed a SPE sample preparation procedure and a LC–MS method for the determination of 5-SCD in human plasma [39]. In the present study, the SPE-LC–MS–MS method was fully validated, including method selectivity and matrix effect evaluations, and was tested in a pilot study. The complete validation taking into account all the prescriptions of the FDA guidelines relative to bioanalytical method validation was successfully performed.

2. Materials and methods

2.1. Chemicals and reagents

5-SCD (97.2%) was graciously offered by NIMH (National Institute of Mental Health, USA). α-Methyldopa (MeD, 99.0%), the internal standard, was supplied by Acros Organics (New Jersey, USA). Hydrochloric acid (37%) and formic acid (98–100%) were supplied by Merck (Darmstadt, Germany). Water and methanol of LC–MS grade were obtained from Biosolve (Valkenswaard, The Netherland). Ascorbic acid was from Certa (Braine-l'Alleud, Belgium) and o-phosphoric acid (min. 85%) from UCB (Leuven, Belgium). All reagents were of analytical grade. Water used in most experiments was of Milli-Q quality (Millipore, Bedford, MA, USA). Oasis MCX sorbents (30 mg/ml) were obtained from waters (Milford, MA, USA). Nitrogen (alphagaz 1) and argon (alphagaz 2) were purchased from Air Liquide (Milmort, Belgium).

2.2. Apparatus

The HPLC system was composed of a quaternary pump, a vacuum degasser, an autosampler and a thermostated column compartment (HP 1100 series, Agilent Technologies, Waldbronn, Germany) controlled by a LC Chemstation. MS detection was carried out using an Ultima triple quadrupole instrument (Waters, UK) operating under MassLynx 3.5 and configured with a Z-spray electrospray ionization source. The separation was performed on an Atlantis dC18 column (100 mm × 2.1 mm, i.d.; particle size: 3 μm) from Waters. A manual extraction system, Manifold® (Merck), was used for the sample preparation during the validation phase; the flow rate applied for the sample loading was set at 0.5 ml of plasma per minute.

All the results obtained during the pre-study validation, the in-study validation as well as the pilot study were treated by the e.noval® and new-daily® softwares (Arlenda, Liège, Belgium).

2.3. Chromatographic conditions

The chromatographic separation of 5-SCD from the internal standard and other catecholamines (cf. [39]), was achieved by gradient elution on an Atlantis dC18 column compatible with mobile phases with very low content of organic modifier. The mobile phase consisted for one part of 0.1% (v/v) formic acid in water and for the other part of 0.1% (v/v) formic acid in methanol. The content of organic modifier increased from 0 to 10% in 10 min. Prior to use, mobile phases were degassed for 15 min in an ultrasonic bath. The chromatographic separation was performed at 20 °C using a constant flow rate of 0.25 ml/min. The injected volume was 20 μl and the thermostatted autosampler was set at 10 °C.

2.4. Extraction technique

Sample preparation was performed by applying solid phase extraction technique on Oasis MCX cartridges. The following optimized procedure was applied: (i) conditioning of the extraction support: the sorbent was treated first by 1.0 ml of methanol and then by 1.0 ml of 0.1 M hydrochloric acid. (ii) Loading of the plasma sample (flow rate: 0.5 ml/min): 1 ml acidified plasma sample was applied on the sorbent. (iii) Washing: the sorbent was successively washed with 1.0 ml of 0.1 M hydrochloric acid and 1.0 ml of methanol. (iv) Elution: 5-SCD and α-methyldopa were eluted from the sorbent with 0.5 ml of the mixture methanol/water/ammonia (80:10:10; v/v/v) containing 0.1% ascorbic acid. This step was followed by the acidification of the eluate with 100 μl of formic acid in order to minimize the analyte oxidation. The eluate was collected and gently evaporated for 1 h under a nitrogen stream. The dried extract was finally reconstituted in 250 μl of water containing 0.1% formic acid and 0.1% ascorbic acid, the solution was submitted to centrifugation and the supernatant was injected into the LC system.

2.5. Mass spectrometry detection

Mass spectrometry detection was carried out using an Ultima triple quadrupole apparatus equipped with an electrospray interface operating in the positive ion mode. 5-SCD and its internal standard were detected by multiple reactions monitoring (MRM) performed on electrospray positive generated ions. The source was heated up to 120 °C and the desolvation temperature was settled at 450 °C. The applied voltages for the capillary and the cone were respectively 3.0 kV and 30 V and the gas flow rates were set at 160 L/h for the cone and 750 L/h for the desolvation. The mass spectrometer generated and selected the pseudomolecular ions [MH^+] for both 5-SCD and α-methyldopa (see Table 1).

Table 1
Followed transitions and values of collision voltage for 5-SCD and the internal standard.

Compound	Precursor ion (m/z)	Fragment ion (m/z)	Collision (eV)	
5-SCD	317	155	30	Quantification
	317	228	15	Identification
α-Methyldopa	212	166	15	

2.6. Standard solutions used for validation phase

An appropriate amount of 5-SCD was dissolved in 10 mM hydrochloric acid solution in order to obtain a concentration of 32 μg/ml (stock solution). Dilutions of the stock solution were performed to reach final concentrations of 64, 192, 384, 4000 and 8000 ng/ml. In the same way, stock and diluted solutions of α-methyldopa were prepared in order to obtain a final concentration of 2000 ng/ml. All diluted solutions were freshly prepared.

Daily, plasma stored at −80 °C was first thawed at ambient temperature in the darkness and centrifuged at 4500 rpm for 10 min. A mixture containing 25 μl of the ascorbic acid stock solution, 25 μl of the 5-SCD solution and 25 μl of the internal standard solution was prepared. Plasma previously acidified by 2% phosphoric acid was then added up to 1 ml and the final sample was mixed. The spiked plasma samples were treated by solid phase extraction. Calibration curves ranging from 1.6 to 200 ng/ml were performed in the matrix, every one by using diluted solutions and each calibration standard was injected in duplicate. In addition, daily, a calibration curve without the matrix was directly injected into the LC–MS–MS system.

3. Results and discussion

In a previous work [38], we optimized and carried out a prevalidation of a SPE-LC–MS/MS method for the determination of 5-SCD in human plasma. The aim of this study was to evaluate the reliability of the method by performing a complete validation according to FDA guidelines and carrying out a pilot study on patient samples.

3.1. Determination of stability

A stability study of 5-SCD and its internal standard (MeD) was performed in order to reflect accurately the situations met during sample handling in all process phases. Stability was evaluated in stock solutions but also in the matrix before and after solid phase treatment, as well as in storage conditions similar to these of the real samples and after three freeze/thaw cycles. Experiments relative to the stability in the matrix were performed using QC at two concentration levels, 4.8 and 200 ng/ml, i.e. three times the lower limit of quantification and the upper end of the concentration range, respectively. A pool of plasma spiked at the two concentrations was prepared, aliquoted and freezed if necessary. For each tested condition, three QC of each concentration level were submitted to the extraction process and analysed by SPE-LC–MS–MS.

3.1.1. Stability of the stock and diluted solutions

Stability of the stock solutions stored at +4 °C was evaluated at UV and MS concentration levels for a period of minimum 4 weeks. Weekly, diluted solutions were prepared from stock solutions and analysed immediately. All peak responses were included between 95 and 105% of the initial value (T_0) attesting the stability of the stock solutions of 5-SCD and MeD in the prescribed conditions.

Stability of the diluted solutions was also investigated at room temperature for a 24 h period of time and the obtained peak responses were comparable to the T_0 ones. The diluted solutions are thus allowed to be used for one day before rejection.

3.1.2. Bench-top and post-preparative stabilities

As a first series of plasma samples was treated and analysed in order to constitute the reference data base (T_0), a second batch was stored at room temperature and in darkness, after the addition of the internal standard and 0.1% of ascorbic acid as anti-oxidative substance. This second series was submitted to the extraction process 4 h after its preparation as prescribed by the FDA [31] and directly analysed. Indeed, a 4 h period of time was sufficient to cover the entire preparation time and the extraction process. Post-preparative stability was evaluated by analysing samples after 24 h in the LC autosampler.

In biological media, cysdopa will be considered stable in the prescribed conditions if the obtained value is comprised between 85 and 115% of the T_0 value. Compared to the initial time (T_0), the mean calculated ratio (±SD) for the low concentration level were 101.3% (±5.4) and 100.3% (±2.8) for $T_{4\,h}$ and $T_{24\,h}$, respectively. Values obtained for the samples at the upper concentration level were 99.9% (±9.6) and 101.8% (±4.5) for the same stability times. These results attest the bench-top and post-preparative stability of 5-SCD in the experimental conditions.

3.1.3. Long-term and freeze/thaw cycles stabilities

In order to meet the recommendations of FDA guidelines for bioanalytical method validation, long-term stability was estimated for a period of time covering sample collection, validation and end of the routine analysis which took place within 4 weeks. QC samples were frozen at −80 °C and analysed weekly. At the end of this period, mean values obtained for the assay (±SD) were 95.6% (±3.0) and 99.3% (±4.2) for the lower and upper concentration levels, respectively.

Three aliquots of QC samples spiked at the two concentration levels were stored at −80 °C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were transferred back to the original freezer at −80 °C and kept frozen for 12–24 h. The cycles of thawing and freezing were repeated twice and the specimens were analysed after the third cycle. Results of the determination (±SD) were 98.9% (±3.7) and 95.9% (±3.9) respectively, confirming the stability of samples for the two concentration levels.

3.2. Method selectivity and matrix effect

Matrix effects were described as phenomenon observed when components issued from the biological matrix coeluted with the analyte of interest, affecting the detection capability, the precision or the accuracy of the method [32]. Evaluation of the matrix effect and its impact on precision, selectivity and/or sensitivity is of utmost importance, especially when a quantitative method is optimized. This is clearly expressed in the FDA and AAPS recommendations [31,33] relative to bioanalytical method validation, particularly when the selected internal standard is not a labelled isotope. Several strategies have been described, giving the opportunity to determine which step of the method is influenced by the matrix presence (i.e. solid phase extraction, MS detection). In this study, two methodologies were retained: post-column infusion as described by Bonfiglio et al. and the determination of the matrix factor [33–36]. Absence of matrix interferences at the retention times and at the selected transitions of 5-SCD and MeD was first evaluated by post-column infusion. A constant quantity of the analytes was delivered in the chromatographic eluant before entering in the MS source. MRM detection of the infused analytes was performed during injection of 6 different blank treated samples. Each endogenous compound which would be eluted and cause a variation in the

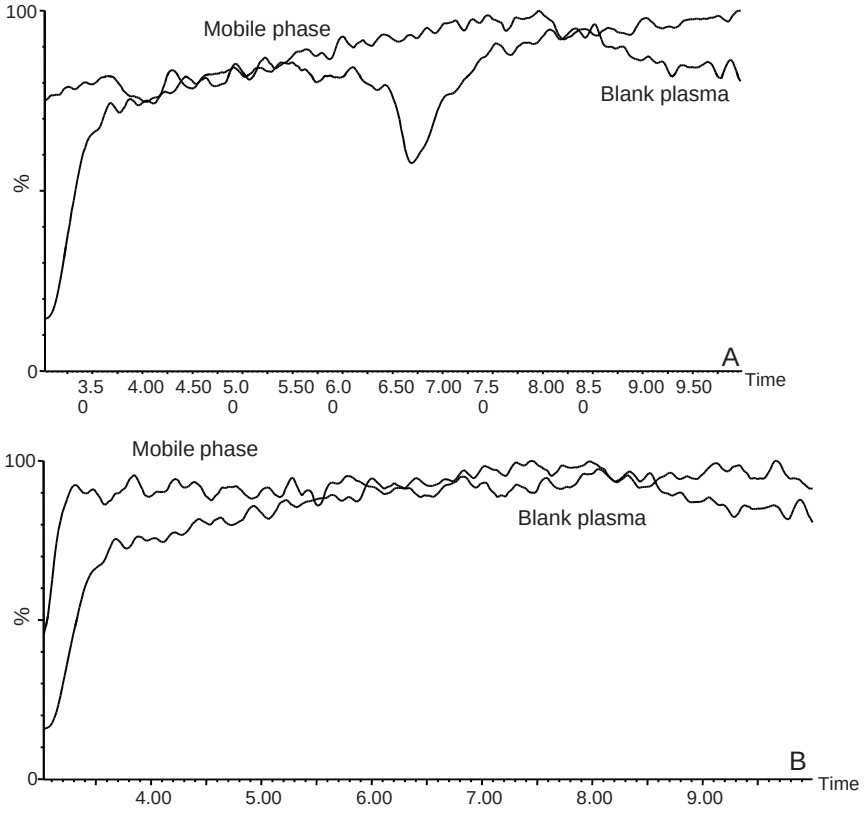

Fig. 2. MS profiles of mobile phase and blank plasma injections during (A) cysdopa and (B) α-methyldopa infusions.

ionisation process of the infused analyte would induce a variation of the response. As illustrated in Fig. 2A and B, no interference was observed at 5-SCD and methyldopa retention times (T_R: 5.2 min and T_R: 6.3 min, respectively). Identical profiles were actually obtained for the other plasma batches (data not shown).

In addition, a quantitative approach for the assessment of the matrix effect was performed in accordance with the recommendations of AAPS report [33]. Accordingly, post-extraction blank samples spiked with 5-SCD and MeD and neat solutions at the same concentration level were analysed. The peak response was the ratio of the peak areas for 5-SCD and MeD. The matrix factor (MF) was defined as:

$$\mathrm{MF} = \frac{A}{B}$$

where A and B are the analyte peak responses in the presence and in the absence of matrix ions, respectively.

A matrix factor equal to 1.00 ± 0.05 (mean ± SD, $n = 6$) was obtained, demonstrating the absence of any matrix effects during LC–MS–MS analysis. One of the most important parameters in the elaboration of a reliable bioanalytical method is not the value of the matrix factor but its variations. Expression of this variability was obtained by the determination of the RSD of all the matrix factors, i.e. 5.5% for this study. This value was clearly under the maximum 15% recommended by the AAPS. Therefore, influence of the matrix on MS detection can be considered as non significant [33,34].

Influence of the matrix on the extraction step can be evaluated by calculating the ratio of the responses obtained when plasma is spiked before or after SPE. Actually, matrix effects on LC–MS–MS step are in this case the same whatever the type of sample is analysed [36]. The obtained ratio being 0.59 ± 0.10, plasma can be considered as influencing significantly the sample preparation phase in this study. This was confirmed by the global recovery of the process which was found lower after application of the plasma samples ($61.6\% \pm 7.1\%$) than the neat samples ($93.7\% \pm 4.0\%$).

3.3. Method pre-study validation

The pre-study validation of an analytical method is mandatory before its routine application in order to ensure its fitness for purpose which is quantifying analytes with a known accuracy [38]. The optimized method being relatively complex, several performances of the analytical procedure were tested before actual validation [39]. During this pre-validation phase, the pertinence of the selected calibration model was evaluated as well as the estimation of the limit of quantification and the dosing range. With this aim in view, the strategy using accuracy profiles based on tolerance intervals for the total error, including both bias and standard deviation for intermediate precision, was followed [40,41]. During the validation step, acceptance limits were enlarged with, in corollary, a risk decrease. The first ones were set up at ±30%, a value accepted by the AAPS guidelines when the validation results are expressed in terms of total error, while the risk was settled at 5%.

3.3.1. Fit for purpose of selecting the calibration curve

The response function of a process represents, within a dosing range, the relationship between the response (i.e. the ratio of 5-SCD and methyldopa area) and the concentration (quantity) of 5-SCD in the considered sample [40]. As observed during the pre-validation [39], the accuracy profile obtained using a quadratic regression weighted by $1/x^2$ offers the best performances in terms of trueness and precision (cf. Fig. 3). In addition, the ability of the method to quantify the compound of interest over the whole concentration range considered is established as the tolerance interval. It is comprised in the acceptance limits over the entire dosing range.

Table 2
Validation results of the analytical method dedicated to the quantification of 5-SCD in human plasma.

Validation criterion	Results (weighted $1/x^2$ quadratic regression, calibration range ($m=4$), 1.6–200 ng/ml)		
	Series 1	Series 2	Series 3
Response function ($k=3$, $n=2$)			
Slope	0.01843	0.01713	0.01990
Intercept	−0.01958	−0.01462	−0.02131
Quadratic term	−0.00000761	0.00000568	−0.00000820
r^2	0.9942	0.9963	0.9988

Validation criterion	Results
Trueness ($k=3$, $n=4$), relative bias (%)	
1.6 ng/ml	0.9
4.8 ng/ml	1.7
9.6 ng/ml	6.2
100 ng/ml	−2.7
200 ng/ml	4.0
Precision ($k=3$, $n=4$), repeatability/intermediate precision (R.S.D.%)	
1.6 ng/ml	6.9/6.9
4.8 ng/ml	7.3/7.4
9.6 ng/ml	8.3/8.3
100 ng/ml	7.9/9.3
200 ng/ml	3.0/4.4
Accuracy ($k=3$, $n=4$), relative β-expectation lower and upper limits (%)	
1.6 ng/ml	[−14.8, 16.7]
4.8 ng/ml	[−15.4, 18.7]
9.6 ng/ml	[−12.9, 25.3]

The calibration curves obtained from the selected model are represented by the following equation:

$$Y = a + bX + cX^2$$

where Y is the analytical response, X the introduced concentration (in ng/ml), a the intercept, b the slope and c is the quadratic term. The regression parameters are presented in Table 2.

3.3.2. Precision and trueness

Precision and trueness are not considered here as decisional parameters but their determination allows assessing the quality of the analytical method as they correspond to random errors and systematic, respectively [40]. As can be seen in Table 2, the overall relative bias does not exceed 6.5% and the relative standard deviations for repetability (intra-day) and intermediate precision are below 10%, all these results being in accordance with the FDA guidelines criteria (i.e. 15%).

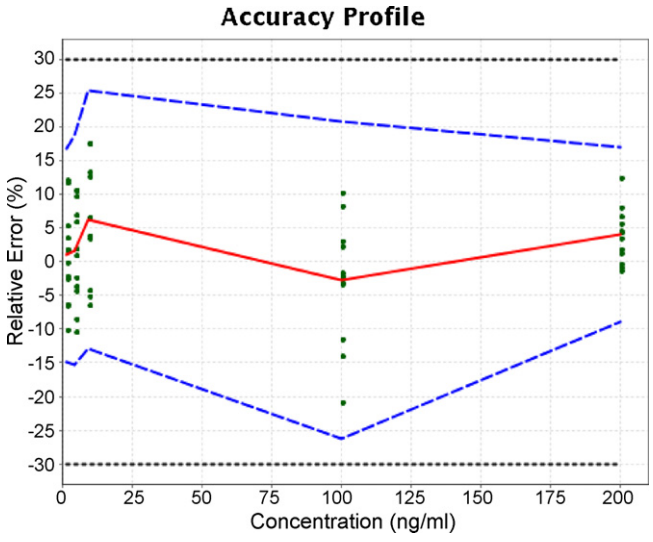

Fig. 3. Accuracy profiles obtained considering a weighted $1/x^2$ quadratic regression: acceptance limits, 30%, risk, 5%. Plain line: relative bias; dashed lines: β-expectation tolerance limits; dotted curves: acceptance limits; dots: relative back-calculated concentrations.

3.3.3. Linearity

The linearity of the results is demonstrated by fitting a regression line on the back-calculated concentrations of the validation standards as a function of the introduced concentrations. Application of a linear regression model based on the least squares method led to the following equation:

$$Y = -0.8168 + 1.032\,X,$$

where Y is the back-calculated concentration (ng/ml) and X is the introduced concentration (ng/ml). The calculated coefficient of determination is equal to 0.9948. All these elements allow attesting the linearity of this analytical method.

3.3.4. Accuracy

The accuracy of the results should be estimated by considering the overall measurement error, i.e. the simultaneous combination of the systematic and random errors, arising from the experimental results [37]. This is achieved by computing the β-expectation tolerance interval or accuracy profiles which guarantees that routine results will be with a minimum risk different from the conventional true values [38]. The method will be declared accurate if β-expectation tolerance limits entirely fit the ±30% acceptance

Table 3
Risk in % of having future measurements falling outside the ±30% acceptance limits in routine analysis for the quantification of 5-SCD in human plasma using the selected response function.

Concentration level (ng/ml)	Risk (%)
1.6	0.17
4.8	0.41
9.6	2.62
100	3.75
200	0.09

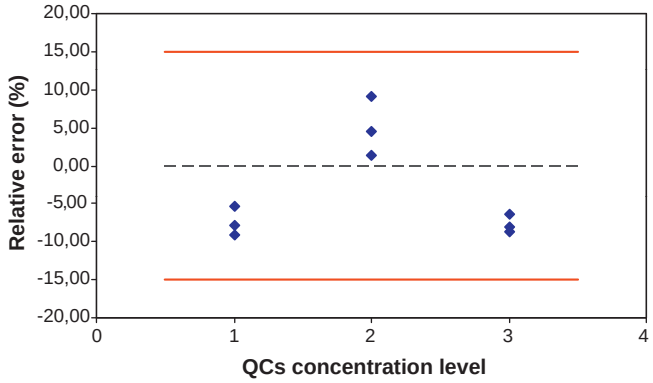

Fig. 4. Results of the in-study validation. QC samples by concentration level (level 1 = 4.8 ng/ml, level 2 = 100 ng/ml and level 3 = 200 ng/ml) of 5-SCD in human plasma in relative values (%). The dots are the relative back-calculated concentrations, the continuous lines are the ±15% acceptance limits for the run and the dotted line is the 0% relative error line.

interval irrespective of the concentration level, as demonstrated in the present application (Table 2).

3.3.5. Risk assessment

As previously stated, the maximum risk $(1-\beta)$ to find future results falling outside the acceptance limits was set up at 5%. The effective probability to obtain such measurements was estimated using accuracy profiles [38]. As can be seen in Table 3, the risk to obtain results outside the acceptance limits set at ±30% is maximum 3.75% for 5-SCD quantification in plasma during routine analysis using the selected response function. It can be noticed that FDA prescription in this matter tolerates a 33% risk, following the 4-6-15 rule [31].

3.3.6. Limits of detection and quantification and evaluation of carry-over

The lower and upper quantification limits are the lowest and highest analyte concentrations which can be reliably determined with an acceptable accuracy under the defined experimental conditions [38]. In the present application, the quantification limits are the extreme concentration levels of the tested dosing range since the accuracy profile, which characterizes the overall measurement error, is confined within the acceptance limits over the whole concentration range (1.6–200 ng/ml). The detection limit estimated on the basis of the mean intercept of the calibration line build in the matrix and the residual variance of the regression [38], was 0.5 ng/ml for 5-SCD.

Absence of any carry-over effect was demonstrated by injecting water immediately after injection of highly spiked samples. No peak could be detected at the retention times and transitions corresponding to 5-SCD and methyldopa.

Finally, absolute recoveries calculated on the basis of data issued from validation are 56.9 ± 9.0, 62.3 ± 7.9, 64.0 ± 4.8 and 59.6 ± 4.8 (mean (%) ± SD) for the following concentration levels, 4.8 ng/ml (n = 12), 20 ng/ml (n = 6), 100 ng/ml (n = 18) and 200 ng/ml (n = 18), respectively.

3.4. Method in-study validation

An in-study validation including 9 QC of 3 concentration levels selected in 4.8–200 ng/ml concentration range was performed during a pilot study of twenty samples. This answers to the in-study validation recommendation edited by the FDA, namely the incorporation of QC at 3 concentration levels minimum into the analysis sequence: the first one corresponding to three times the LLOQ, the second in the middle of the selected concentration range and the third one at the ULOQ (namely 4.8, 100 and 200 ng/ml). The response function applied for the results determination is a quadratic regression weighted by $1/x^2$ which was predisposed to offer the best performances in terms of accuracy [31].

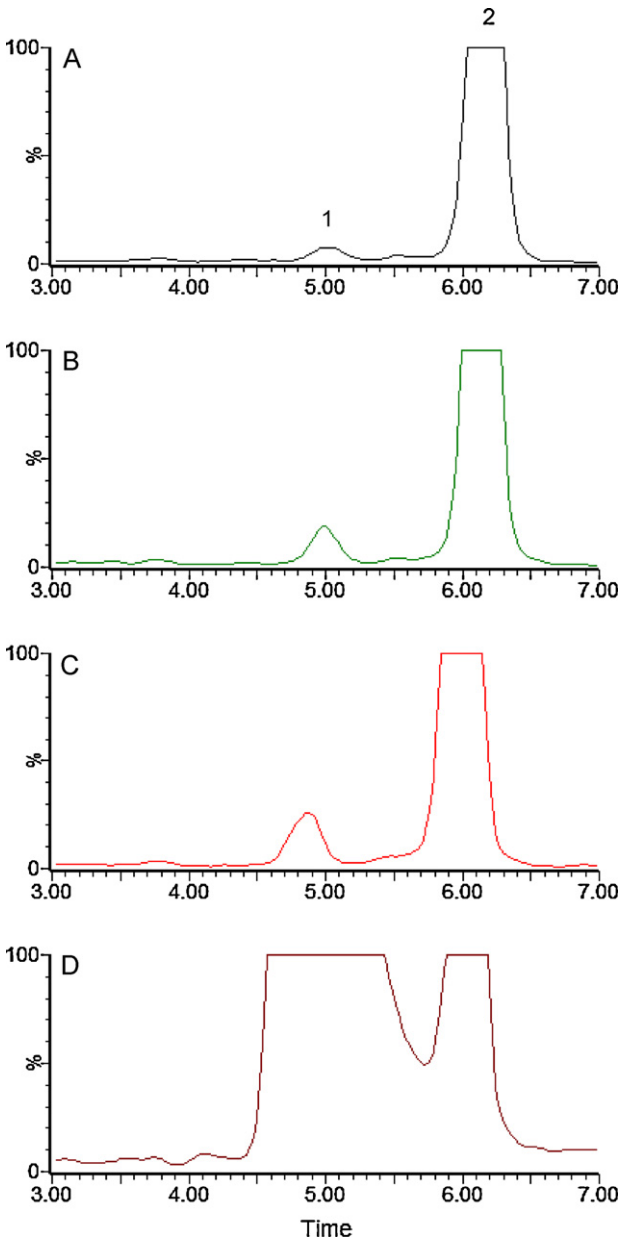

Fig. 5. Examples of chromatogram for plasma samples obtained after SPE-LC–MS–MS process: peak 1, cysdopa; peak 2, α-methyldopa. (A) Blank plasma (pool of controls) with internal standard; (B) plasma at the LLOQ; (C) a negative control sample; (D) a positive melanoma patient sample in terminal phase (5-SCD >200 ng/ml).

Back-calculated QC values allow establishing acceptability of the series of experiments following FDA recommendations, namely 67% of the QC must be in the acceptance limits set at ±15%. As this criterion was filled, the series of analysis can be accepted (Fig. 4).

3.5. Pilot study

This work was finalized by carrying out a pilot study involving determination of plasma samples. Ten patients with melanoma treated at CHU of Liège and five control subjects were included

in this preliminary study. All the patients and controls gave their informed consent for the search and the assay of serum melanoma markers.

Examples of chromatogram obtained during this study is presented in Fig. 5. Plasma 5-SCD levels obtained for control individuals were all inferior to 2.5 ng/ml (below the pathologic threshold which is generally set at 3.2 ng/ml). Samples from 10 patients were analysed and only one value superior to 200 ng/ml (ULOQ) was found. This corresponded to the plasma sample of a patient in an advanced stage of the disease. 7 others were found to have 5-SCD levels inferior to the 3.2 ng/ml limit. From a clinical point of view, these patients were either in remission stade (5) or treated by chemotherapy (1) or presented adjuvant melanoma without metastasis (1). The low 5-SCD value obtained in case of chemotherapy could be explained by the fact that 5-SCD levels should decrease up to normality after a successful treatment. Finally, the 2 last patients of this pilot study either carried a high number of naevi or an intensive sunshine likely to explain the observed 5-SCD levels, 3.2 and 4.5 ng/ml respectively. As all these results were promising, additional analysis of samples from patients with intermediate or advanced stage of this pathology should be ideal in order to test more completely the method. After that, a large scale study should be envisaged.

4. Conclusions

This paper describes the pre-study and in-study validation of a LC–MS/MS method for the determination of 5-SCD in human plasma.

An original strategy, based on total measurement error and accuracy profiles as a decision tool, allowed to demonstrate that the method was reliable for its intended use over a dosing range comprised between 1.6 and 200 ng/ml. The maximum risk of observing future measurements falling outside the acceptance limits during routine analysis was below 4%. All the validation results complied with the FDA recommendations.

From the results obtained during the in-study validation, the method applicability was demonstrated, since all results of the QC samples were included in their respective β-expectation tolerance intervals.

Acknowledgements

Research grants from the Belgium National Fund for Scientific Research (FNRS) to two of us (M.F. and A.-C.S.) are gratefully acknowledged.

References

[1] N.J. Ives, R.L. Stowe, P. Lorigan, K. Wheatley, J. Clin. Oncol. 25 (2007) 5426–5434.
[2] V. Bataille, E. de Vries, BMJ 337 (2008) 1287–1291.
[3] C.M. Balch, A.C. Buzaid, S.J. Soong, M.B. Atkins, N. Cascinelli, D.G. Coit, I.D. Fleming, J.E. Gershenwald, A. Haughton, M. Kirkwood, K.M. McMasters, M.F. Mihm, D.L. Morton, D.S. Reintgen, M.I. Ross, A. Sober, J.A. Thompson, J.F. Thompson, J. Clin. Oncol. 19 (2001) 3635–3648.
[4] J. Hartleb, R. Arndt, J. Chromatogr. B 764 (2001) 409–443.
[5] M.P. Di Cesare, A. Antunes, F. Truchetet, Encycl. Méd. Chir., Dermatologie, 98-595-A-10, Scientifiques et Médicales Elsevier, Paris, 2000.
[6] C. Thirlwell, P. Nathan, BMJ 337 (2008) 1345–1348.
[7] J. Utikal, D. Schadendorf, S. Ugurel, Arch. Dermatol. Res. 298 (2007) 469–477.
[8] S. Medic, R.L. Pearce, P.J. Heenan, M. Ziman, Pigment Cell. Res. 20 (2006) 80–91.
[9] T. Nakastura, T. Kageshita, S. Ito, K. Wakamastsu, M. Monji, Y. Ikuta, S. Senju, T. Ono, Y. Nishiruma, Clin. Cancer Res. 10 (2004) 6612–6621.
[10] K. Stoitchkov, T. Le Bricon, Ann. Biol. Clin. 58 (2000) 157–165.
[11] F. Egberts, A. Pollex, J.H. Egberts, K.C. Kaehler, M. Weichenthal, A. Hauschild, Onkologie 31 (2008) 380–384.
[12] S. Kruijff, E. Bastiaannet, A.C. Muller Kobold, R.J. van Ginkel, A.J. Suurmeijer, H.J. Hoekstra, Ann. Surg. Oncol. (2009), 28 july 2009 published online: springerlink.com.
[13] I. Salama, P.S. Malone, F. Mihaimeed, J.L. Jones, EJSO 34 (2008) 357–364.
[14] S.J. Ohsie, G.P. Sarantopoulos, A.J. Cochran, S.W. Binder, J. Cutan. Pathol. 35 (2008) 433–444.
[15] A.R. Larson, E. Konat, R.M. Alani, Nat. Clin. Practice Oncol. 6 (2009) 105–117.
[16] S. Medic, R.L. Pearce, P.J. Heenan, M. Ziman, Pigment Cell Res. 20 (2006) 80–91.
[17] A.K. Bosserhoff, Pigment Cell Res. 18 (2005) 411–416.
[18] V. Bucetic, S.A. Rogan, P. Hrabac, N. Hudorovic, H. Cupic, L. Lukinac, M. Ledinsky, A. Matejcic, I. Lovricevic, M. Zekan, Melanoma Res. 18 (2008) 201–207.
[19] M.A. Hofmann, F. Gussman, A. Fritsche, S. Biesold, B. Schicke, I. Küchler, C. Voit, U. Trefzer, Melanoma Res. 19 (2009) 17–23.
[20] K. Koyanagi, S.J. O'Day, R. Gonzales, K. Lewis, W.A. Robinson, T.T. Amatruda, C. Kuo, H.J. Wang, R. Milford, D.L. Morton, D.S.B. Hoon, Clin. Cancer Res. 12 (2006) 1137–1143.
[21] J. Rangel, M. Nosrati, S. Torabian, L. Shaikh, S.P.L. Leong, C. Haqq, J.R. Miller, R.W. Agebiel, M. Kashani-Sabet, Cancer 112 (2008) 144–150.
[22] B. Vucetic, S.A. Rogan, P. Hrabac, N. Hudorovic, H. Cupic, L. Lukinac, M. Ledinsky, A. Matejcic, I. Lovricevic, M. Zekan, Melanoma Res. 18 (2008) 201–207.
[23] F. Buback, A.C. Renki, G. Schulz, J.M. Weiss, Exp. Dermatol. 18 (2009) 750–759.
[24] L. Brochez, J.M. Naeyaert, British J. Dermatol. 143 (2000) 256–268.
[25] T. Horikoshi, S. Ito, K. Wakamatsu, H. Onodera, H. Eguchi, Cancer 73 (1994) 629–636.
[26] K. Wakamatsu, T. Kageshita, M. Furue, N. Hatta, Y. Kiyohara, T. Ono, T. Saida, N. Takata, T. Tushida, H. Uhara, A. Yamamoto, N. Yamazaki, A. Naito, S. Ito, Melanoma Res. 12 (2002) 245–253.
[27] T. Banfalvi, K. Gilde, M. Boldizsar, Z. Fejös, B. Horvath, G. Liszkay, E. Beczassy, T. Kremmer, Eur. J. Clin. Invest. 30 (2000) 900–904.
[28] E. Kvam, J. Dahle, Cancer Lett. 221 (2005) 131–134.
[29] R. Wolber, K. Schlenz, K. Wakamatsu, C. Smuda, Y. Nakanishi, V.J. Hearing, S. Ito, Pigment Cell Melanoma Res. 21 (2008) 487–491.
[30] K. Arstrand, A. Kullman, R. Andersson, T. Rasmuson, B. Kagedal, Scand. J. Clin. Lab. Invest. 64 (2004) 559–564.
[31] Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Reseach (CDER), Center for Biologics Evaluation and Research (CBER), May 2001.
[32] P. Kebarle, L. Tang, Anal. Chem. 65 (1993) 972A–986A.
[33] C.T. Viswanathan, S. Bansal, B. Booth, A.J. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, R. Weiner, AAPS J 9 (2007) E30–E42.
[34] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019–3030.
[35] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, Rapid. Commun. Mass Spectrom. 13 (1999) 1175–1185.
[36] I. Marchi, S. Rudaz, J.L. Veuthey, J. Pharm. Biomed. Anal. 49 (2009) 459–467.
[37] ISO document: ISO 5725, Accuracy (trueness and precision) of measurement methods and results, Parts 1–4, 6, ISO, Geneva, Switzerland, 1994.
[38] M.A. Bimazubute, E. Rozet, I. Dizier, J.C. Van, E. Heugen, P. Arancio, J. Gustin, P. Crommen, Chiap, J. Chromatogr. B 877 (2009) 2349–2357.
[39] G.B. Martin, P. Chiap, Ph. Paquet, G. Pierard, P. de Tullio, Y. Martin, E. Rozet, Ph. Hubert, J. Crommen, M. Fillet, J. Chromatogr. A 1156 (2007) 141–148.
[40] Ph. Hubert, J.J. Nguyen-huu, B. Boulanger, E. Chapuzet, P. Chiap, N. Cohen, P.A. Compagnon, W. Dewe, M. Feinberg, M. Lallier, M. Laurentie, N. Mercier, G. Muzard, C. Nivet, L. Valat, STP Pharma Pratiques 13 (2003) 101–138.
[41] S. Rudaz, S. Souverain, C. Schelling, M. Deleers, A. Klomp, A. Norris, T.L. Vu, B. Ariano, J.L. Veuthey, Anal. Chim. Acta 492 (2003) 271–282.