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Determination of Homovanillic Acid and Vanillylmandelic Acid in Neuroblastoma Screening by Stable Isotope Dilution GC-MS

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A method for the quantitative determination of homovanillic acid (HVA) and vanillylmandelic acid (VMA), two metabolites of catecholamines, is presented. The assay is based on gas chromatography/electron impact mass spectrometry. The preparation of ^{13}C-labeled VMA from [$^{13}C_6$]vanillin is described. Together with purchased deuterated HVA the ^{13}C-labeled VMA is used as an internal standard in stable isotope dilution GC/MS. The method involves elution from soaked filter-papers, determination of creatinine content, extraction of HVA and VMA from eluted and urinary samples and derivatization to the di-and tri(trimethylsilyl) derivatives, respectively. The detection limits were found to be 4.0 pg for HVA and 0.8 pg for VMA. The method was applied to the routine determination of urinary HVA and VMA in a range from 5 to 100 ng HVA and VMA per μg creatinine. The lower limits of pathological concentrations are set at 35 ng μg^{-1} creatinine for HVA and to 20 ng μg^{-1} creatinine for VMA, which are in close correlation with the values from other methods, but with the main advantage of reducing the amount of questionable or elevated results from 6.7% (high-performance liquid chromatography (HPLC) alone) to 0.9% (HPLC and GC/MS).

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INTRODUCTION

Neuroblastoma, a neuroblastic tumor,[1] is the most frequent extracranial solid tumor in early childhood. In Austria, one out of 7600 children is affected by this disease.[2,3] The most frequent tumor sites are the pre-and paravertebral sympathetic ganglia and the adrenal gland.[1]

If detected early enough,[4] neuroblastoma is a curable tumor. Without screening, 65% of the tumor patients are diagnosed with advanced disease (stage III (tumor extending in continuity beyond the midline of the body) or stage IV according to Evans' criteria [remote disease involving the skeleton, bone marrow, soft tissue and distant lymph node groups[1])]. The prognosis of patients in these stages is very poor despite of a very aggresive therapy including bone marrow transplantation or peripheral stem cell transplantation. The 5 year survival rate is less than 50%.[5] The prognosis of tumor patients in stages I (tumor confined to the organ) and II (tumor extending in continuity beyond the organ, but not crossing the midline), however, is favorable and results in a 5 year survival rate of more than 90% after resection of the tumor.

Early diagnosis of the tumor can be done by determination of various tumor markers excreted continously in elevated amounts in the urine of infants at ages between 7 and 10 months.

Neuroblastoma, derived from primordial neural crest, delivers epinephrine (adrenaline), norepinephrine (noradrenaline) and dopamine. Dopamine and other metabolites of catecholamines, mainly homovanillic acid (HVA) and vanillylmandelic acid (VMA), are excreted continuously in higher concentrations even in the early stages of the tumor.[6–8]

In 1985, a screening program was started in Japan, measuring HVA and VMA concentrations in the urine of children at the age of 6 months. The positive results of the Japanese studies induced several other countries in Europe and America to start similar projects.

In 1990, a screening program was started in Austria, measuring tumor markers in the urine of children at the age of 7–10 months.[3,7] At first screening was performed by determination of the HVA and VMA concentrations in buffered urine by an enzymatic imunoassay (EIA) method.[7] However, being expensive and of relative low reproducibility, the EIA method was then replaced by a more accurate high-performance liquid chromatographic (HPLC) method.[3]

In our program for neuroblasroma screening, HPLC is now used as the primary method. About 89% of all received samples show a clear negative result by use of HPLC analysis alone. About 4% of all samples are insufficient (low urine content on filter-paper, etc.), whereas more than 6% show questionable or elevated values, in many cases a result of co-eluting, unidentified

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peaks on the HPLC trace. All samples with elevated values of HVA and/or VMA are again tested, after elution in buffer and determination of the creatinine content, by stable isotope dilution gas chromatography/electron impact mass spectrometry (GC/EIMS).[9–11] Using [$^{13}C_6$]VMA and [$^{2}H_5$]HVA (d_5-HVA) as internal standards, quantitation is carried out by comparing the ratio of the peak areas with those of the calibration curve. By use of GC/MS as a secondary method, the rate of questionable or elevated results can be reduced to less than 1%.

Together with the synthesis of $^{13}C_6$-labeled VMA, a quick and stable method has been developed for routine determination of urinary HVA and VMA concentrations, which is used as an independent control in neuroblastoma screening and therefore reduces the need for retesting samples from about 11% (HPLC alone) to about 5%.

EXPERIMENTAL

Materials

HVA, VMA (HPLC screening standard) and Lyphochek Quantitative Urine Control were obtained from Bio-Rad (Germany), HVA also from Sigma (Germany), VMA also from Serva (Germany), $^{2}H_5$-labeled HVA from Promochem (Germany), $^{13}C_6$-labeled vanillin from CIL (Andover, MA, USA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine from Pierce (Rockford, IL, USA). All other solvents and reagents of analytical grade were obtained from Merck (Darmstadt, Germany).

Gas chromatography/mass spectrometry

A Fisons Model 8000 gas chromatograph coupled to a Fisons MD 800 quadrupole mass spectrometer was used. The column was directly connected to the ion source of the mass spectrometer. The gas chromatograph was equipped with a DB-5MS fused-silica capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness) from Fisons. The splitless Grob injector was kept at 260 °C. Helium was used as the carrier gas with an inlet pressure of 50 kPa. The initial column temperature was 70 °C, held for 1 min, followed by an increase at 30 °C min^{-1} to 230 °C and an isothermal hold of 2 min (GC oven parameter settings; the actual time needed to reach 230 °C is 6.5 min). The transfer line between the GC and MS systems was kept at 260 °C. The ion source temperature was 200 °C.

Electron impact mass spectra were recorded with an electron energy of 70 eV and an emission current of 100 μA.

Synthesis of ^{13}C-labeled vanillylmandelic acid[12–14] ([$^{13}C_6$]VMA)

The synthesis of ^{13}C-labeled vanillylmandelic acid ([$^{13}C_6$]VMA) was carried out as shown in Fig. 1 and described in the literature.[12–14] A 50 mg portion of vanillin-ring-$^{13}C_6$ (0.33 mM, 85% isotopic enrichment) was converted into 3-methoxy-4-hydroxymandelonitrile by the use of potassium cyanide and sodium hydrogensulfite in water. This compound was isolated as a slightly yellowish oil and treated with dry hydrogen chloride and ethanol, forming the corresponding iminoester hydrochloride. After saponification with sodium hydroxide and acidification with dilute HCl, the labeled VMA was isolated as a colorless oil.

The reactions were monitored by thin-layer chromatography (TLC), using silica and chloroform–methanol–water (65:35:5) as eluent and staining the TLC plates by heating to a high temperature. The reaction product was also checked by GC/MS (scan mode) and a single peak was visible in the chromatogram. The [$^{13}C_6$]VMA was compared with a commercial natural analog and showed complete identity, as well as by TLC and GC/MS, with the exeption of the molecular mass (see also Fig. 2).

Sample elution and creatinine determination

As described previously,[9] the urine of children at the age of 7–10 months is collected by placing a piece of filter-paper in the infant's diaper, where it is soaked by the urine. After drying in air, it is mailed to the laboratory. A piece of about 1 cm^2 is cut off, eluted with 0.5 ml of phosphate buffer (pH 3) overnight at 4 °C and the creatinine content of the solution is determined using the method of Jaffé (see below). The HVA and VMA concentrations are then measured by HPLC and calculated as μg per mg creatinine.[3] Creatinine is used as a reference to measure the amount of urine that is soaked by the filter-paper.

All samples with elevated HVA and/or VMA levels or with outstanding HPLC traces are again tested by GC/MS in the following manner. A second piece of filter-paper is eluted in the same way as above and the creatinine content is again estimated according to Jaffé: 50 μl of blank (0.01 M HCl in H_2O) and calibration

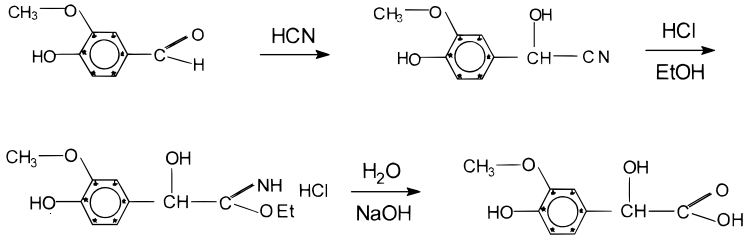

Figure 1. Synthesis of $^{13}C_6$-labeled vanillylmandelic acid (VMA) starting from labeled vanillin. For details, see text and Refs 12–14.

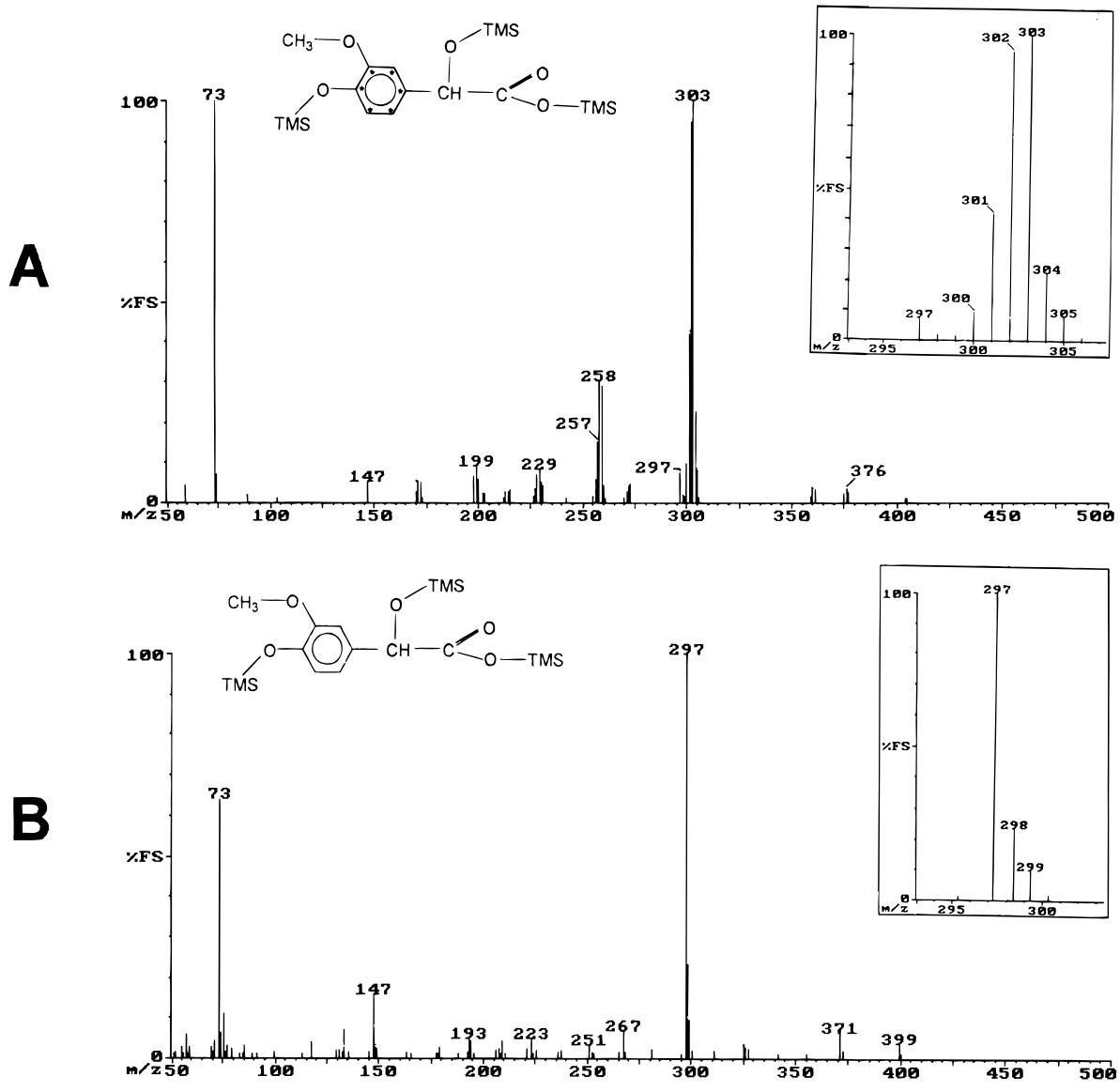

Figure 2. EI mass spectra, isotopic composition (inset) and molecular structures of (A) $^{13}C_6$-labeled VMA and (B) unlabeled VMA after derivatization with BSTFA. Labelling and derivatization were carried out as described in the text.

standards (in duplicate) and 50 µl of the eluted samples (and also of urinary samples) are placed in cell culture cluster dishes. A 50 µl volume of a freshly prepared 1:1 mixture of picric acid (22 mM) in water and sodium hydroxide solution (0.75 M) are added and the plate is shaken. After 1 h at room temperature, sample absorption is measured in a microplate reader (Model 3550-UV from Bio-Rad, Vienna, Austria) at a wavelength of 490 nm and the creatinine concentration of the samples is calculated, together with the exact volume of the eluate (or urine) that contains 1 µg creatinine.

Sample preparation and derivatization with BSTFA

A 50 µl portion of the internal standard solution (containing 20 ng of [$^2H_5$]HVA and 20 ng of [$^{13}C_6$]VMA in methanol) is added to a volume of eluted sample (or urine, when children are checked in the hospital) corresponding to 1 µg of creatinine (normally between 5 and 100 µl of eluted puffer). This solution is acidified with 0.5 ml of dilute HCl (2 ml of concentrated HCl in 1 l of H_2O), to which 4 g of cobalt(II) chloride per liter are added (for better visualization of the two liquid phases). This slightly pink solution (2 mg of $CoCl_2$ per sample) is saturated with NaCl (~200 mg per sample) and extracted with 2.5 ml of ethyl acetate. After centrifugation, the supernatant is decanted and the solvent is evaporated under a stream of nitrogen. 50 µl of BSTFA in pyridine (2:1, v/v) are added and the sealed vials are incubated at 75 °C. After 1 h the vials are cooled to room temperature and the solution is diluted with 200 µl of hexane, transferred into autosampler vials and an aliquot of 2 µl is subjected to GC/MS analysis.

Analytical method validation

Creatinine. Calibration graphs for creatinine were established in the range 5–100 mg l^{-1}. Inter-assay variations

(mean ± standard deviation of 1σ, unless indicated otherwise) were determined by carrying five identical buffer samples at concentrations of 85 and 112 mg l^{-1} through the analytical procedure. Intra-assay variations (mean ± s.d.) were estimated by analysing two samples at concentrations of 72 and 104 mg l^{-1} ten times with the microplate reader.

HVA and VMA. Calibration graphs were established in the range of 5–100 ng of HVA and VMA per sample. The detection limit was estimated by derivatizing adequate amounts of unlabeled and a constant amount of labeled HVA and VMA in the manner described above and injecting aliquots of 2 µl into the GC/MS system. The inter-assay variations (mean ± s.d.) were determined by carrying five identical urine samples at concentrations of 11 and 76 ng HVA and of 5 and 32 ng VMA per sample through the analytical procedure. Intra-assay variations (mean ± s.d.) were estimated by analysing three samples at concentrations of 2, 18 and 79 ng of HVA and 1, 20 and 34 ng of VMA per sample ten times by GC/MS.

To optimize the conditions for the elution of the compounds from the filter-paper into the buffer, series of eluted (4 °C, overnight) samples were worked up as usual, stored under different conditions (deep freezer, refrigerator and room temperature, with and without filter-paper inside, for 1 week), processed again and the results were compared.

Freeze–thaw properties were tested by immediate analysis of Lyphocheck control urine and after ten freeze–thaw cycles. The concentrations used were creatinine at 104 and 112 mg l^{-1}, HVA at 16 and 85 ng per sample and VMA at 28 and 76 ng per sample.

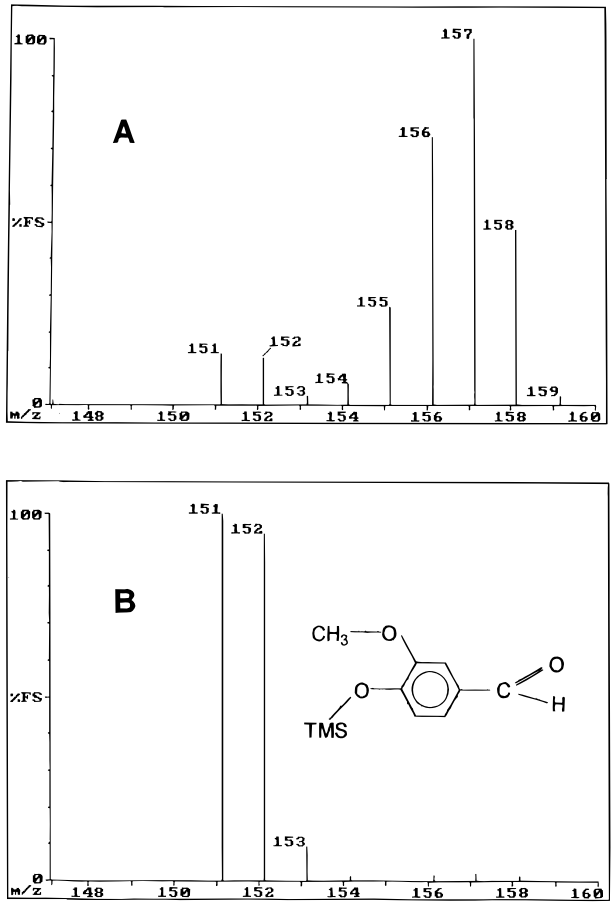

Figure 3. Isotopic abundance of (A) [$^{13}C_6$]vanillin and (B, base peaks of mass spectra) the natural analog after derivatization with BSTFA and GC/MS analysis, as described in the text.

RESULTS AND DISCUSSION

Synthesis of ^{13}C-labeled vanillylmandelic acid ([$^{13}C_6$]VMA)

As described earlier,[9] VMA can be measured together with HVA and deuterated HVA without labeled internal VMA standard. However, the physical and chemical properties of HVA and VMA are different, which can cause difficulties in the quantitation of the substances. This and the fact that no suitable stable isotope-labeled VMA was available made it important to synthesize labeled VMA in our laboratories. A possible method of preparation[12–14] is shown in Fig. 1, starting from vanillin, which is available in different stable isotope-labeled forms. We decided to use a $^{13}C_6$-ring-labeled vanillin (3-methoxy-4-hydroxybenzaldehyde) for synthesis, representing a difference in the molecular mass between natural and labeled VMA of 6 u.

The first synthesis yielded about 0.5 mg of [$^{13}C_6$]VMA. A second preparation gave 10 mg of [$^{13}C_6$]VMA (from 200 mg of vanillin) with the same isotopic enrichment as the vanillin [85%, as can be seen by comparing the inset in Fig. 2(A) with Fig. 3(A)]. The reaction product (vanillylmandelic acid) was compared with a commercial natural analog and also by TLC [chloroform–methanol–water (65:35:5)] and GC/MS (scan mode). Both substances showed single-peak chromatograms in TLC and identical chromatograms in GC with comparable mass spectra and fragmentations (as trimethylsilyl derivatives; see Fig. 2).

Sample preparation and derivatization with BSTFA

As can be seen in Fig. 5, the single-ion recording (SIR) EI mass chromatogram of a patient's urine, treated as shown above, with labeled standards and derivatized with BSTFA, is very clean. Owing to the five deuterium atoms, replacing five hydrogen atoms in the labeled HVA molecule (see also molecular structures in Fig. 4), the [$^2H_5$]HVA standard elutes a short time earlier than the unlabeled compound (isotope effect). This effect can be observed to a greater extent when appropriate GC parameters are used, as shown by Marchese and Caruso.[11] On the other hand, this procedure increases the GC time up to 30 min, which is not the aim of a screening method where large numbers of samples have to be measured (in our case more than 10 000 GC/MS runs per year). The addition of $CoCl_2$ (2 mg per sample) to the aquous phase before extraction of HVA and VMA into ethyl acetate had no influence on the results. Controls (20 samples) have been made with and without

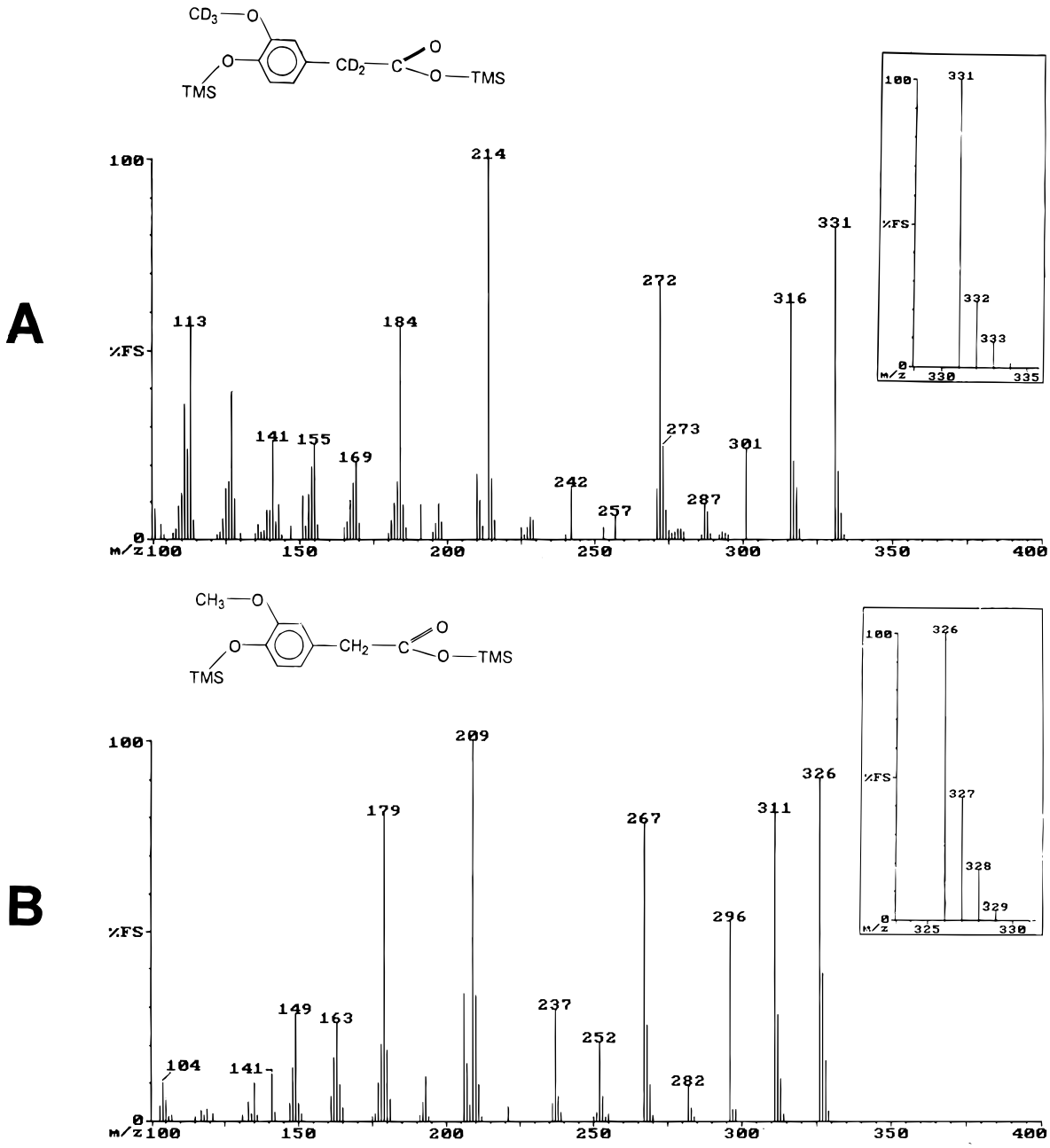

Figure 4. EI mass spectra, isotopic composition (inset) and molecular structures of (A) deuterium-labeled HVA and (B) unlabeled HVA after derivatization with BSTFA. Derivatization was carried out as described in the text.

$CoCl_2$ addition and the HVA and VMA concentrations were within the standard deviations of measurement.

The corresponding mass spectra of (A) labeled and (B) unlabeled VMA and HVA are shown in Figs 2 and 4, respectively. As can be seen in the spectra of VMA (Fig. 2), the base peaks are at m/z 297 (B) and m/z 303 (A), respectively, resulting from α-activation and benzylic cleavage ($[M - COOTMS]^+$), whereas practically no molecular ions (m/z 414 and 420, respectively) are visible. The isotopic compositions of the base peaks are shown in the insets in Fig. 2. The fact that there is also an amount of 10% of unlabeled VMA present in the spectrum of labeled VMA is due to impurities in the ^{13}C-labeled vanillin. For visualization of this inconvenience, we checked the $[^{13}C_6]$vanillin(3-methoxy-4-hydroxybenzaldehyde) together with the natural analog by GC/MS. The GC traces of the trimethylsilyl derivatives (prepared and scanned in the same way as shown above) of the two compounds are identical. The isotopic composition of the base peaks of (B) unlabeled and (A) $[^{13}C_6]$vanillin are shown in Fig. 3. The base peaks are at m/z 157 ($[^{13}C_6]$vanillin) and m/z 151, respectively, resulting from TMS elimination from the molecule, whereas practically no molecular ions (m/z 224 and 230, respectively) are visible. Comparison of the isotopic composition of $[^{13}C_6]$vanillin [Fig. 3(A)] with the isotopic composition of $[^{13}C_6]$VMA [inset in Fig. 2(A)] shows nearly the same isotopic abundance in both compounds. This inconvenience, however, is corrected by calibration graphs (see below). As can be seen in the

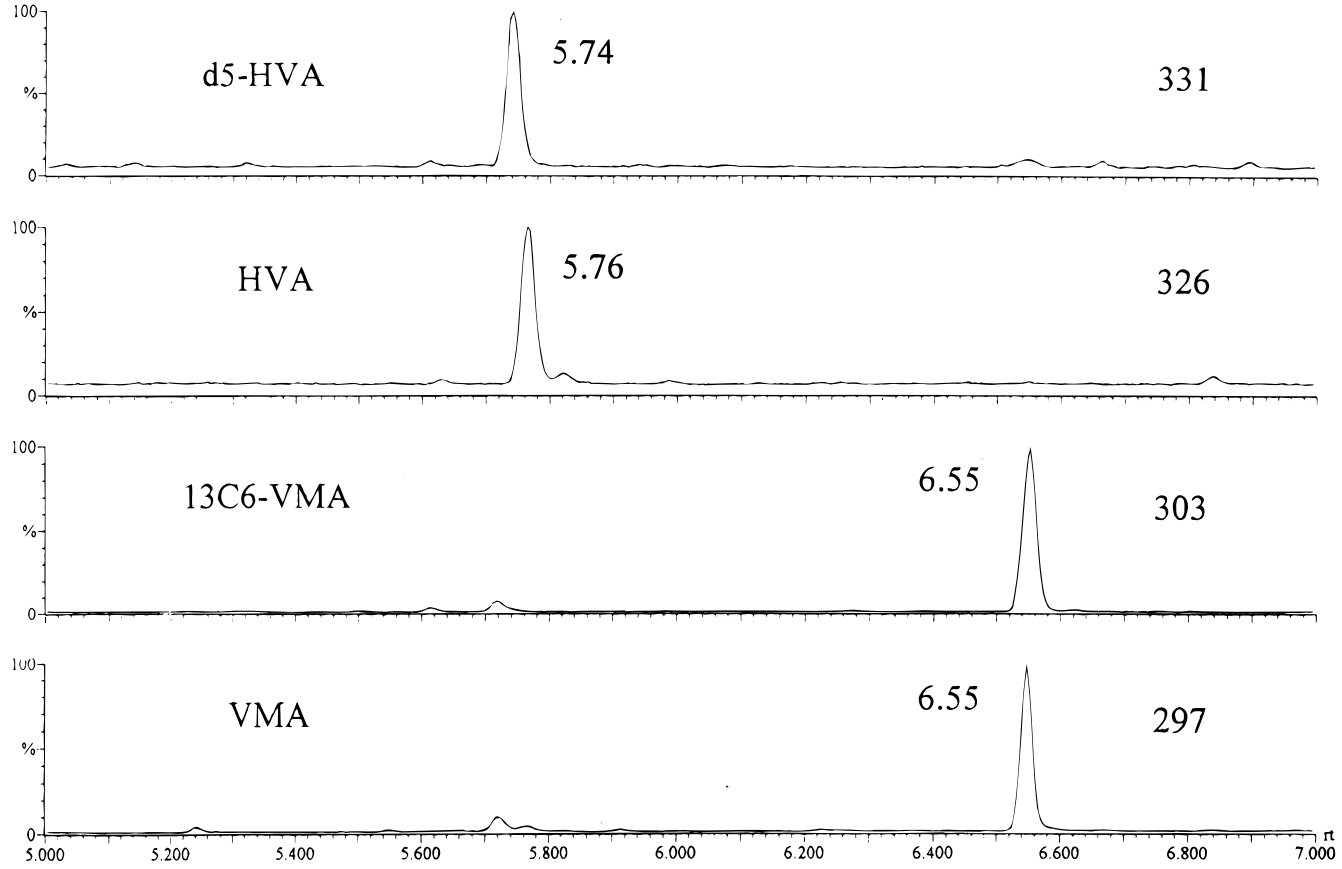

Figure 5. Typical SIR EI mass chromatogram of a urine sample from a human volunteer, treated with labeled HVA and VMA and derivatized with BSTFA, as described in the text. The amount analysed corresponds to 23 ng HVA per μg creatinine (m/z = 326) and to 16 ng VMA per μg creatinine (m/z = 297), whereas the standard concentration of both compounds was 20 ng μg^{-1}.

spectra of HVA (Fig. 4), the molecular ion at (B) m/z 326 and (A) m/z 331, respectively, shows nearly the same abundance as the ions resulting from benzylic cleavage [base peak at m/z 209 (unlabeled) and m/z 214 (labeled)]. The isotopic composition of the molecular ion again is shown in the insets in Fig. 4.

Analytical method validation

Creatinine. The calibration graph established was linear within the range 5–100 mg of creatinine per liter of eluate (r = 0.9998). Inter-assay variations (mg of creatinine per liter of eluate ± s.d.) were estimated to be 85.02 ± 7.55 and 111.96 ± 3.7. Intra-assay variations (mg of creatinine per liter of eluate ± s.d.) were estimated to be 71.9 ± 0.08 and 104.5 ± 0.23.

HVA and VMA. The calibration graph (see also Fig. 6) established was linear within the range 5 –100 ng HVA and VMA per sample. The linear regression for VMA (r = 0.99984) was calculated as follows: peak area of natural compound divided by peak area of isotope (ordinate) = 0.00376A + 0.03358B × concentration of VMA (abscissa). The regression data for HVA are r = 0.99955, A = 0.00367 and B = 0.05552. The data shown are from a typical routine experiment.

The deviation of the ratio of the peak areas from theoretical values (as can be seen in Fig. 6) is caused by differences in the actual (isotopic) amount of labeled compounds, but is corrected by the calculation of HVA and VMA concentrations. As discussed earlier, there is a certain amount (~10%) of unlabeled VMA present in the spectra of labeled VMA. To reduce the possible influence of this "contamination" on samples with low VMA concentrations, in general we reduce the quantity of internal standard added. However, in this case we decided to add an amount of 20 ng of labeled VMA, because the main interest with this screening program is to detect samples with elevated results and the cut-off limit for pathological concentrations of VMA is set to 20 ng. Furthermore, our control samples, as well as the Lyphocheck controls, are at the same concentration. The HVA and VMA concentrations of all samples are calculated in the same manner as described above, which means that only the ratio of the peak areas is responsible for the concentration of the compounds. To minimize errors of weighing and pipetting, we analyze all samples in duplicate.

The limits of detection were found to be 4.0 pg for HVA (actually injected) and 0.8 pg for VMA at a signal-to-noise ratio of at least 4:1, whereas the limits of detection were set to 2 ng per ml of eluate (or urine) for HVA and 1 ng per ml of eluate for VMA. For special experiments there would be a possibility of using even more eluate for extraction, but in our routine experiments the concentrations of the compounds of interest would exceed the calibration range. Inter-assay variations

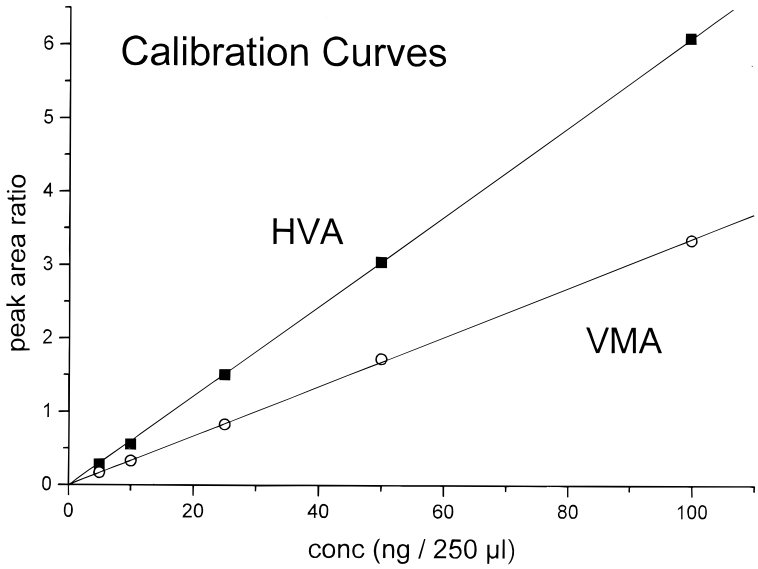

Figure 6. Calibration graphs for HVA and VMA, as used in the quantitation of neuroblastoma samples. For details, see text.

(mean ng per sample ± s.d.) were estimated to be 11.32 ± 0.27 and 76.29 ± 1.93 for HVA and 4.91 ± 0.07 and 32.17 ± 0.50 for VMA, respectively. Intra-assay variations (mean ng per sample ± s.d.) were estimated to be 1.95 ± 0.05, 17.64 ± 0.25 and 79.07 ± 2.07 for HVA and 1.11 ± 0.05, 20.06 ± 0.35 and 33.61 ± 0.43 for VMA, respectively.

Routine determinations were run as described in the Experimental section. Testing the elution process for optimum results showed that, after elution overnight at 4 °C, storing the eluates with and without filter-paper in sealed vials at 4 °C the concentrations of creatinine, HVA and VMA remain unchanged within the s.d. limits of measurement. If the samples are frozen with filter-papers inside, the creatinine content is reduced slightly (−9%), whereas the HVA and VMA concentrations increase by 10% (mean of all tested samples after 1 week of deep freezing). If the samples are stored at room temperature it is very important to seal the vials (results unchanged within s.d. limits), otherwise the results are irreproducably changed, mainly owing to growth of bacteria.

After ten freeze–thaw cycles Lyphocheck urine control standards showed reduced concentrations of creatinine, HVA and VMA. Of original creatinine concentrations of 110 and 102 mg l^{-1}, only 85 and 75% were found, respectively. For initial HVA concentrations of 16 and 92 ng per sample, 97 and 75% were recovered, and for VMA concentrations of 31 and 71 ng per sample, 99 and 75% were recovered.

HVA and VMA concentrations are expressed in ng per µg of creatinine (as reference for urine concentration on filter-paper) owing to the different amounts of urine that have actually soaked the filter-paper.

To safeguard against system variations, we decided to use the following routine program. After running three blank samples (for cleaning the GC/MS system), ten calibration samples (5, 10, 25, 50 and 100 ng of each of

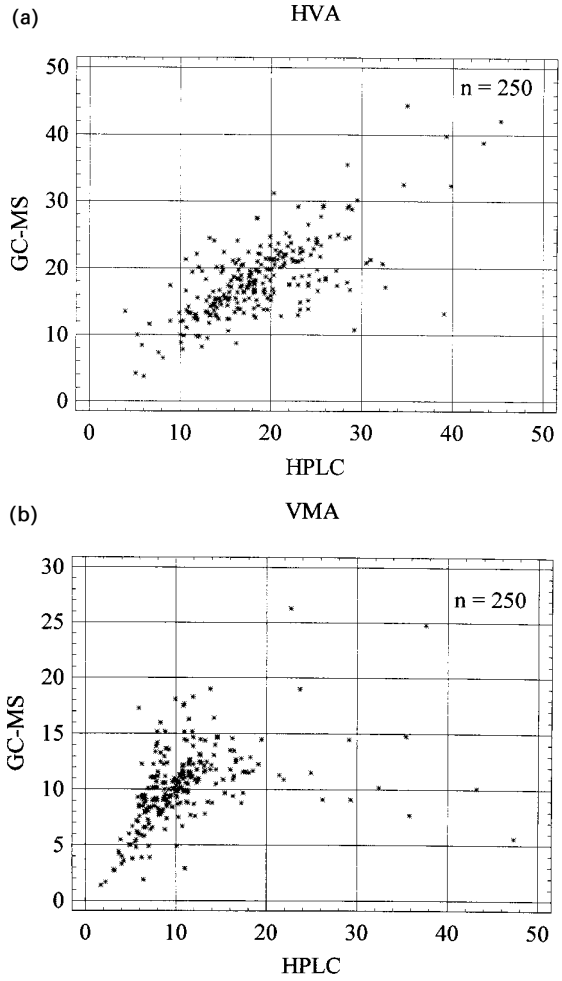

Figure 7. Scatter plot of 250 randomly selected urine samples analyzed by HPLC and GC/MS for (A) HVA and (B) VMA. Values are given in ng μg^{-1} creatinine. Several samples show elevated values for VMA by the HPLC method (>20 ng), but values in the normal range by the GC/MS method (B).

HVA and VMA, in duplicate) are measured, followed by another blank. Then two control samples (20 ng of HVA and VMA) are analyzed, followed by 35 buffer eluates of urine samples from 35 children (in duplicate). After these 72 samples (prepared together in one run), another four control samples (Lyphocheck urine control standard, normal and pathological values, in duplicate) are subjected to GC/MS. Calibration standards and Lyphocheck controls are prepared and checked before being used. Each of these series represents a single batch that is calculated in the manner described above. A macro has been written to automate the quantitation, so that only the baseline of the corresponding peaks has to be set. When the concentration of all controls are within their s.d. limits ($\pm 1\sigma$) the values for the samples are accepted. Through this validation there is no need for further corrections, as was shown in previous work.[9,11]

We then investigated 250 randomly selected eluted urine samples from healthy infants of the relevant age group to find the limits of pathological concentration (see Fig. 7). The results showed that the cut-off limits (mean value + 2.5 s.d.) have to be set at concentrations of 35 ng μg^{-1} creatinine for HVA and 20 ng μg^{-1} creatinine for VMA. These are in close correlation with the values from the HPLC method, which has been confirmed by a paired t-test ($P = 0.94$ for HVA and 0.3979 for VMA; do not reject the null hypothesis for $t = 0.05$).

Several samples showed elevated values [>20 ng VMA per μg creatinine, Fig. 7(B)] for VMA by the HPLC method, but these values were in the normal concentration range when double-checked by the GC/MS method.

Because of its capability of measuring a large number of samples in a relative simple, cheap and accurate way, HPLC now is used as the primary method in our program for neuroblasroma screening. We have found that 89.1% of all samples show a clear negative result by HPLC analysis alone; 4.2% of all samples are insufficient (low urine content on filter-paper, stool contamination, insufficiently dried filter-paper, etc.), and 6.7% show questionable or elevated (mainly VMA) values, in many cases a result of co-eluting, unidentified peaks. By use of GC/MS as a secondary method, the rate of questionable or elevated results can be reduced to 0.9%. Therefore, by using HPLC as a primary and GC/MS as a secondary method only 5.1% of all samples received have to undergo a retest, whereas a retest would be necessary in 10.9% of cases without the use of GC/MS.

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REFERENCES

1. G. M. Brodeur and R. P. Castleberry, in *Principles and practice of pediatric oncology*, ed. by P. A. Pizzo and D. G. Poplack, 2nd edn, p 739. J. B. Lippincott, Philadelphia (1989).
2. R. Ladenstein, Ch. Urban, H. Gadner, F. M. Fink, A. Zoubek, W. Emminger, H. Grienberger, K. Schmitt, P. F. Ambros, I. M. Ambros, E. Horcher, G. Amann, G. Höfler, R. Kerbl and I. Mutz, *Eur. J. Cancer*, **31A**, 637 (1995).
3. R. Kerbl, Ch. Urban, Ch. Schellauf, Ch. Sitzwohl, H. Lackner, H. Gleispach, G. Fauler, P. F. Ambros, I. M. Ambros, S. Rumpler, H. Gadner, E. Spuller, G. Höfler, G. Amann, J. Kytir and I. Mutz, Wien. Klin. Wochenschr. **108**, 398 (1996).
4. M. Nishi, H.Miyake, T. Takeda, M. Shimada, N. Takasugi, Y. Sato and J. Hanai, Cancer **60**, 433 (1987).
5. M. I. Bernstein, J. M. Leclerc, G. Bunin, L. Brisson, L. Robison, J. Shuster, T. Byrne, D. Gregory, G. Hill and G. Dougherty, *J. Clin. Oncol.* **10**, 323 (1991).
6. M. Nishi, H. Miyake, T. Takeda, H. Yonemori, J. Hanai, Y. Kikuchi and N. Takasugi, Oncology, **48**, 31 (1991).
7. R. Kerbl, Ch. Urban, I. Mutz, P. F. Ambros and H. Gadner, in *Proceedings of the 3rd international symposium on neuroblstoma screening, Kyoto, Japan, September 29–October 1, 1993*, ed. by T. Sawada, T. Matsumara and T. Kizaki, p. 30. Kyoto (1994).
8. T. Itoh and K. Ohmori, *J. Lab. Clin. Med.*, **81**, 887 (1973).
9. H. Gleispach, E. Huber, G. Fauler, R. Kerbl, Ch. Urban and H. J. Leis, Nutrition **11**, 604 (1995).
10. J. A. Seviour, A. C. McGill, G. Dale and A. C. Craft, *J. Chromatog.*, **432**, 273 (1988).
11. N. Marchese and U. Caruso, Chromatographia **36**, 179 (1993).
12. J. A. F. Gardner and H. Hibbert, *J. Am. Chem. Soc.*, **66**, 607 (1944).
13. K. N. F. Shaw, A. McMillan and M. D. Armstrong, *J. Org. Chem.* **23**, 27 (1958).
14. M. D. Armstrong, A. McMillan and K. N. F. Shaw, *Bio Biophys. Acta*, **25**, 422 (1957).

