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THE DETERMINATION OF HOMOVANILLIC ACID IN HUMAN AND RAT URINE USING GAS CHROMATOGRAPHY WITH FLAME IONIZATION AND MASS SPECTROMETRIC DETECTION

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Abstract—New gas–liquid chromatographic (GLC) methods were developed for the assay of homovanillic acid (HVA) in human and rat urine. The methods are based on the use of extraction and efficient column purifications (silicic acid and lipophilic Sephadex) before quantitative GLC analysis using either a flame ionization detector (FID) with 3-methoxy-4-hydroxy-phenylpropionic acid as internal standard or mass spectrometric (MS) detection using a deuterated HVA derivative as carrier and internal standard. The precision was found to be ±3–5 per cent (S.D.) for the GLC–FID method and ±10 per cent (S.D.) for the GLC–MS method. The main advantage over existing gas chromatographic methods is about a 100-fold increased sensitivity which allows accurate quantitation of normal or decreased levels of HVA.

PREVIOUSLY available methods for quantitation of homovanillic acid (HVA) are based on fluorometry[1–3] or gas–liquid chromatography (GLC) with flame ionization detection.[4–8] Fluorometric measurement of HVA is relatively unspecific and in a biological fluid as complex as urine this can be a considerable disadvantage. The GLC methods also suffer from some drawbacks, the main one being insufficient sensitivity. Consequently, these methods have been used mostly when the levels of HVA are high, such as in urine from patients with neuroblastoma[4–8] and in the urine of patients receiving l-DOPA therapy.[6–9] In the present paper we report methods for the analysis of HVA allowing determination of normal and decreased levels in human and rat urine.

EXPERIMENTAL

Chemicals. HVA was purchased from Sigma Chemical Co. Its analogue, 3-methoxy-4-hydroxyphenylpropionic acid (HVA-CH_2), was prepared as described previously.[10] HVA-7-^{3}H (sp. act. 1·05 Ci/mmole) was purchased from New England Nuclear Corp., Boston, Mass. U.S.A.

Ethereal diazomethane was prepared by the reaction of *N*-nitrosotoluene-4-sulfomethylamide (Merck AG., Germany) with potassium hydroxide. The solution was stored at − 20° and was used within 5 days of preparation.

Gas–liquid chromatography. The instrument used was a Hewlett–Packard Model 402 gas chromatograph equipped with two glass columns connected to flame ionization detectors (FID). The columns were 1830 × 4 mm and were packed with 3·75% XE-60

on 80–100 mesh Chromosorb W. Nitrogen was used as the carrier gas. The flow rate was 50 ml/min and the pressure was 3 kg/cm^2. For preparation of the columns, the support was coated with the liquid phase using the fluidization technique.[11] Columns were conditioned at maximum temperature for a minimum of 24 hr.

Gas chromatography—mass spectrometry. The identities of the compounds analyzed by FID were verified by gas chromatographic–mass spectrometric analysis on a LKB Model 9000 combined gas chromatograph–mass spectrometer. The instrument was equipped with a 2 m 1% SE-30 column. The ionizing current was 60 μA and the energy of the electrons 22·5 eV. The compounds were collected after passage through a 3·75% XE-60 column and then dissolved in ethyl acetate for injection into the SE-30 column of the LKB instrument.

For quantitative mass spectrometric analysis the deuterated methyl ester of HVA (HVA-CD_3) was used as an internal standard and carrier as described previously.[10] The magnetic current and the accelerating voltage alternator were adjusted to focus alternately on *m/e* 392 and 395, the molecular ions and base peaks of the heptafluorobutyryl (HFB) derivatives of HVA-CH^3 and HVA-CD_3 respectively.[13]

Preparation of samples

Human urine, GLC-FID analysis. To 10 ml of urine was added 10 ml of a saturated solution of NaCl, 50 μg of internal standard (HVA-CH_2) and ^{3}H-HVA (0·1 μCi). The ^{3}H-HVA was used only in initial experiments so that losses occurring during the procedure could be determined. The pH was adjusted to about 2 with 6 N HCl and the sample was extracted three times with equal volumes of ether. After the extraction, water was removed from the ether phase by freezing in a mixture of ethanol and dry ice and filtering under suction. The ether was evaporated in a rotary evaporator. The sample was then reconstituted in 0·5 ml of chloroform in preparation for silicic acid chromatography.

One g of acid washed, activated silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa., U.S.A.) was prepared as a slurry in chloroform and used to make a column with final dimensions of 90 × 5 mm. After the sample had passed into the silicic acid, the column was washed with 5 ml of chloroform and then eluted with 12 ml of a mixture of chloroform and ether (50:1). The silicic acid column proved to be very efficient in removing background material from the samples. Before silicic acid chromatography the dry weight of the residue from ten human urine samples (10 ml urine per sample) was 26·6 ± 8·0 mg. After elution from the column the dry weight of the samples was 1·5 ± 0·9 mg.

The eluate from the column was evaporated under a stream of nitrogen. The sample was then dissolved in 0·25 ml of methanol and ethereal diazomethane was added. The tubes were sealed and allowed to stand overnight. This procedure results in the formation of the methyl ester and methyl ether of HVA and the internal standard (HVA-CH_2).[4] After evaporation under a stream of nitrogen the sample was reconstituted in 0·05 ml of ethyl acetate and analyzed by GLC using a 3·75% XE-60 column. Quantitation was achieved through determination of peak height ratios for the derivatives of HVA and HVA-CH_2 and comparison of these values with a standard curve such as the one shown in Fig. 1.

Rat urine, GLC-FID analysis. Twenty-four hr rat urines were collected in metabolic cages. The urines were diluted to 20 ml with a saturated solution of NaCl, ^{3}H-HVA

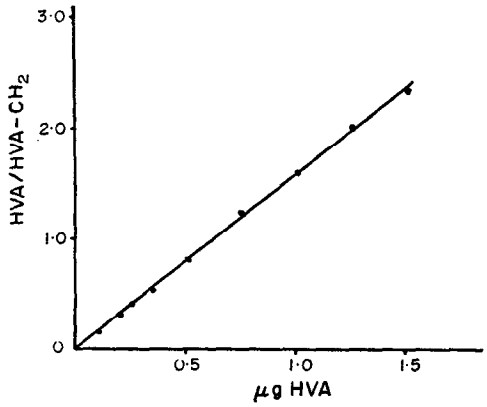

FIG. 1. Response of the flame ionization detector to samples containing methyl ester/methyl ether derivatives of HVA and HVA-CH_2. Peak height ratio is on the ordinate and concentration of HVA (μg) on the abscissa.

(0·1 μCi) was added and the pH was adjusted to 2 with 6 N HCl. The samples were extracted four times with equal volumes of ether. The ether phases were combined, dried, evaporated and then purified by passage over silicic acid columns as was described for human urine.

After formation of the methyl ester/methyl ether derivative as described above, the sample was dissolved in toluene (0·05 ml) and put on a lipophilic Sephadex[12] column (370 × 4 mm) which had been prepared with toluene as the solvent. The sample was eluted with toluene and the fractions containing the radioactivity were collected and combined. The flow rate for the columns was 0·1 ml/min and 0·6 ml fractions were collected. A chromatogram is shown in Fig. 2. The fractions corresponding to the HVA-derivative were combined.

The sample was divided in half and an aliquot of one half was taken to determine radioactive recovery. To the other half was added 15 μg of methyl ester/methyl ether derivative of HVA-CH_2 which acted as an internal standard for the GLC

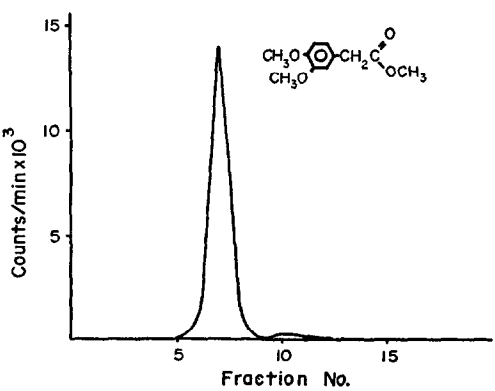

FIG. 2. A chromatogram of the methyl ester/methyl ether derivative of HVA on lipophilic Sephadex. The column dimensions were 370 × 4 mm. The flow rate was 0·1 ml/min and the fraction size was 0·6 ml.

analysis. Each half of the sample was evaporated to dryness and reconstituted in ethyl acetate (0·01 ml) and analyzed by GLC-FID on a 3·75% XE-60 column. Quantitation was performed as described above for human urine with the exception that the results was adjusted with values for recoveries through the extraction and column steps.

*Human and rat urine, GLC-MS analysis.** To 1 ml of human or rat urine was added 1 ml of saturated NaCl solution and about 0·02 μCi of ^{3}H-labelled HVA. After acidification to pH 2–3 with ten drops of N HCl extraction was performed three times with 2 ml of ethyl acetate. The extract was evaporated to dryness under a stream of nitrogen. The dried residue was dissolved in 0·5 ml of $CHCl_3$ and applied to a silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa., U.S.A.) column (5 × 90 mm). After washing the column with 5 ml of $CHCl_3$ the HVA fraction was eluted with 12 ml of 3% diethyl ether in $CHCl_3$. After evaporation to dryness under nitrogen the residue was dissolved in 2 ml of ethyl acetate. Following the removal of 0·1 ml for measurement of radioactivity recovery, 0·2 ml (human urine) or 1 ml (rat urine) was combined with 5 μg of the deuterated methyl ester of HVA (HVA-CD_3). The HVA-CD_3 was added from a stock solution containing 10 μg/ml in 96% ethanol.

The extract was treated briefly with an excess of ethereal diazomethane and the solvent and reagent removed at once by a stream of nitrogen in a fume hood. Then dried residue was reconstituted in 0·05 ml of ethyl acetate and 0·05 ml of heptafluorobutyric anhydride added. After 30 min at room temperature the solvent and reagent were evaporated. The sample was dissolved in 0·5 ml of 0·5% heptafluorobutyric anhydride in ethyl acetate prior to the quantitative analysis by GLC–MS.

The GLC–MS analysis was performed essentially as described previously.[10] In the mass spectrum of the heptafluorobutyrylated HVA-methyl ester (HVA-CH_3-HFB) the molecular ion is the base peak and occurs at a *m/e* value of 392.[13] Other abundant peaks occur at 333 (loss of carbomethoxy) and 197 (loss of heptafluorobutyryl). The mass spectrometer was adjusted by means of the multiple ion detector to focus alternately on 392, due to the endogenous protium compound (HVA-CH_3-HFB), and on 395 which arises from the exogenous deuterium labelled internal standard (HVA-CD_3-HFB). A standard curve is constructed by measuring the peak height ratios of known amounts of HVA-CH_3-HFB in the presence of a constant amount of HVA-CD_3-HFB. The peak height ratio in the unknown samples was measured and the amount of HVA present in the sample could thus be calculated. This figure was corrected for losses suffered through the isolation procedure using the figure obtained for radioactive recovery.

RESULTS

GLC–FID analysis of HVA

Human urine. A representative chromatogram from human urine is shown in Fig. 3. The identity of the derivatives of HVA and HVA-CH_2 that are represented by the indicated peaks in the chromatogram were confirmed by gas chromatography–mass spectrometry.

The precision of the method was evaluated by means of repeated analysis of a pool of human urine. The mean of 7 determinations was found to be 3·70 μg/mg of

* A simplified GLC-MS analysis in urine has now been achieved using pentadeufero-HVA or bisdeufero-HVA as the internal standard (B. Sjöquist, B. Lindström and E. Änggård, to be published).

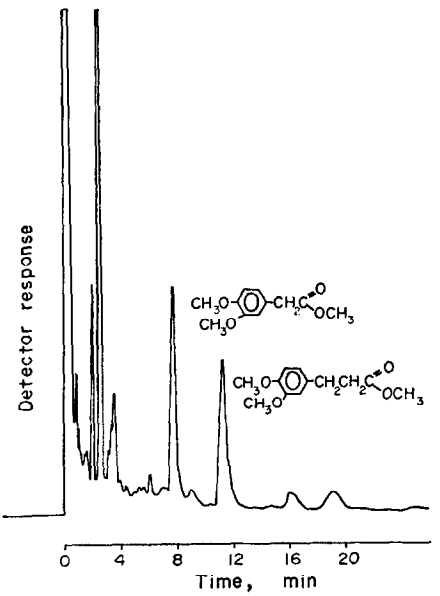

FIG. 3. A chromatogram from human urine containing 3·20 μg HVA/mg of creatinine. The temperatures employed were 155°, 200° and 250° for the column oven, flash heater and detector respectively. The structures shown were confirmed by mass spectrometry.

creatinine with a standard deviation of ±3 per cent. The recovery of added HVA was found to be 96·9 ± 1·6 per cent (mean ± *SD*) when 25 μg of HVA was added to 10 ml of urine and 95·9 ± 1·0 per cent (mean ± *SD*) when 50 μg of HVA was added ($n = 4$ in each case).

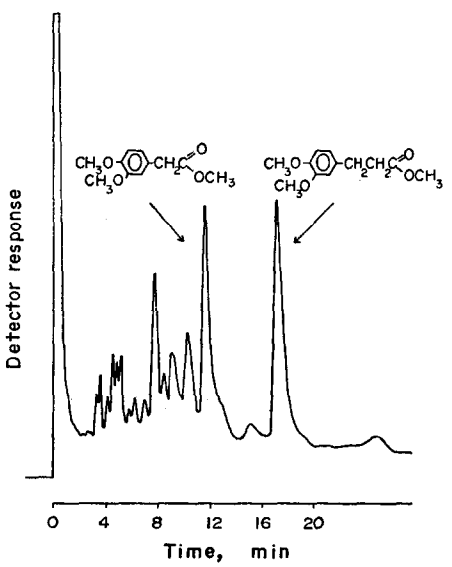

FIG. 4. A chromatogram from rat urine containing 12·0 μg HVA/5 ml rat urine. Conditions as in Fig. 3. The structures shown were confirmed by mass spectrometry.

The recovery of ^{3}H-HVA through the procedure was 69·5 ± 7·1 per cent (n = 12). This should not be confused with the recovery of added HVA which should be very close to 100 per cent if the HVA and the HVA-CH_2 behave identically through the procedure. The radioactive recovery is indicative of parallel losses of HVA and HVA-CH_2. The level of HVA in the urine of 10 healthy volunteers was found to be 3·42 ± 1·72 μg/mg creatinine with a range of 2·04–7·31 μg/mg creatinine.

Rat urine. A representative chromatogram from rat urine is shown in Fig. 4. The structures shown were confirmed by gas chromatography–mass spectrometry.

The precision of the method was determined by repetitive analyses of a pool of rat urine. The mean of six determinations was 2·44 ± 0·12 μg/ml of rat urine. The recovery of added HVA was found to be 97·6 ± 6·1 per cent when 5 μg HVA was added to 5 ml of urine and 90·4 ± 10·4 per cent when 10 μg of HVA was added (n = 4 in each case). The recovery of ^{3}H-HVA through the procedure was 50·1 ± 5·5 per cent (n = 12).

GLC–MS analysis of HVA

Representative chromatograms for the analysis of human and rat urine are shown in Fig. 5. It is apparent that the prior purification by silicic acid chromatography, the relative high levels of HVA in urine and the high specificity of the mass spectrometric detection of this derivative of HVA combines to give chromatograms that are essentially uncontaminated by any other peaks. That the peaks shown in Fig. 5 were due to HVA-CH_3-HFB was shown by recording complete mass spectra of the effluent in samples where no HVA-CD_3 had been added. At the expected retention time of HVA-CH_3-HFB peaks appeared at m/e values of 195 (22 per cent, loss of HFB), 333 (37 per cent, loss of carbomethoxy) and 392 (100% M^+).[13] The relative intensities of these fragments were the same as those in a mass spectrum of authentic HVA-Me-HFB analyzed at the same time.[13]

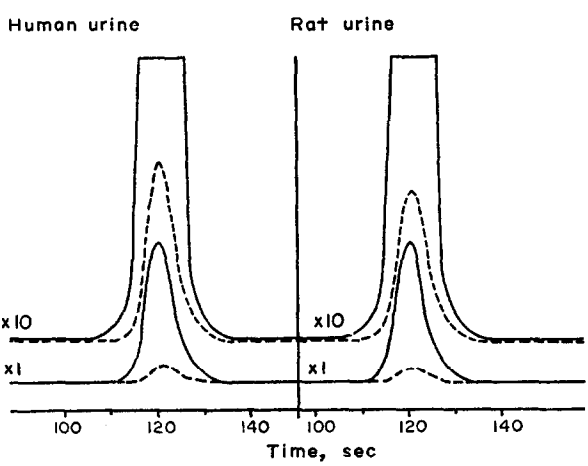

FIG. 5. GLC–MS analysis of HVA in human urine (left) and rat urine (right). The responses from the added deuterated internal standard (HVA-CD_3-HFB) at a m/e of 395 (——) and that of the endogenous compound (HVA-CH_3-HFB) at a m/e of 392 (– – –) are shown at two amplifications. The isolation of the samples from urine as well as instrumental conditions are described under Experimental.

The precision of the method was evaluated using repetitive ($n = 6$) analyses on a pool of urine collected from several normal human individuals. The value obtained was $11{\cdot}9 \pm 1{\cdot}2$ μg/mg of creatinine. A pool of rat urine similarly analyzed gave a value of $1{\cdot}71 \pm 0{\cdot}18$ μg/ml ($n = 6$).

The sensitivity of the GLC–MS is very high and not fully utilized in the analysis of urine. When analyzing urine from normal individuals the signals due to HVA-CH_3-HFB were about thirty times over the background. The sensitivity could be increased further about 100-fold by modifying the amounts of internal standard and volumes of solvent. In practice this is done in the analysis of HVA in cerebrospinal fluid[10] and in serum[14] where the levels of HVA are in the region of 10–50 ng/ml.

DISCUSSION

The present methods are based on extraction of the urine and purification by column chromatography before quantitation by either GLC–FID or GLC–MS. The main advantage over previous GLC-methods[4–8] is the increased specificity and sensitivity permitting the quantitation of normal and reduced levels of HVA. These advantages are achieved in two ways. Firstly, the introduction of the silicic acid purification step eliminates a great deal of interfering compounds, in particular hippuric acid and dihydroxy-phenylacetic acid. Secondly, the GLC–MS technique is inherently more specific and sensitive since it only measures compounds which have the retention time of HVA-Me-HFB and which also have a high intensity fragment at *m/e* value of 392.

It should be mentioned here that the choice of derivatives in the two techniques is of considerable importance. With the GLC–FID method we preferred the methyl ester/methyl ether derivative described by Williams and Sweeley,[5] since it is more stable than perfluoroacylated and trimethylsilylated derivatives and also separates better from the background material on both the lipophilic Sephadex and on the XE-60 column. With the GLC–MS technique the choice of the heptafluorobutyryl derivative[13] is important since the molecular ion constitutes the base peak and occurs at a relatively high mass, where background contributions from other compounds and column bleed are minimal. Another important feature of the HFB derivatives is that fluorine is monoisotopic and the separation between protium and deuterated derivatives is therefore more clearcut.

The precision is relatively high ($SD = 3$–5 per cent) with the GLC–FID method. With the GLC–MS method the precision is at the moment lower ($SD = 10$ per cent). The major factor contributing to the lower precision appears to be the focusing of the mass spectrometer. Small drifts in the magnetic current may move the peaks off the slit and thus cause alterations in the ratio between protium and deuterium derivatives. It is possible that computer guided focusing may improve the precision in the future.

In summary we have presented two alternative GLC methods for the determination of normal and reduced levels of HVA in human and rat urine. Both methods have high specificity and acceptable precision. The GLC–FID is slightly more laborious but requires on the other hand less costly instrumentation and could therefore be used in most well-equipped research laboratories. The GLC–MS is more specific and also faster but has the disadvantage of high cost. These methods are presently being used

together with new GLC–MS techniques for the determination of HVA in cerebrospinal fluid,[10] serum[14] and brain[15] to study the function of the dopaminergic nervous system in man and experimental animals.

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