THE USE OF ISOMERIC COMPOUNDS AS INTERNAL STANDARDS FOR QUANTITATIVE MASS FRAGMENTOGRAPHY OF BIOLOGICAL SAMPLES. DETERMINATION OF HOMOVANILLIC ACID IN CEREBROSPINAL FLUID AND PLASMA

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Received November 13,1973

SUMMARY

A simple method for the determination of homovanillic acid (HVA) in biological samples by the technique of single ion monitoring (mass fragmentography) during gas chromatography-mass spectrometry is described. Iso-homovanillic acid (iso-HVA) and 4-methoxymandelic acid (MMA), two compounds isomeric with HVA, are used as the internal standards. The levels of HVA and iso-HVA were determined by this method in the cerebrospinal fluid of the human and dog.

INTRODUCTION

Sweeley *et al.* (1) first described the technique of selective ion monitoring (SIM), also known as mass fragmentography, in 1966. Since that time SIM or a modification of SIM, multiple ion monitoring, has been widely used for the quantitation of metabolites in the nanomole and picomole range.

The techniques are based on the preparation of suitable derivatives sufficiently volatile for gas chromatographic-mass spectrometric (GC-MS) analysis. For accurate quantitative measurement by GC-MS, it is necessary to use an internal standard. The ideal standard should be as similar as possible to the compound under analysis, so that the yields across the separator for sample and standard are the same. This is generally achieved in conventional GC-MS by using a homologue or a closely related compound with a slightly different retention time as an internal standard. This technique is illustrated in the detection of dopamine, indoleacetic acid and nortriptyline by GC-MS. These compounds were derivatized to pentafluoropropionate (2), heptafluorobutyryl methyl ester (3) and trifluoroacetamide (4), respectively. The ideal internal standard is an isotopically labelled derivative of the test compound, for the fragmentation patterns and the retention times will be the same. However, some fragments will contain one

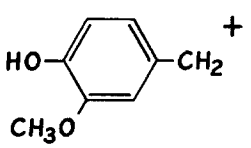
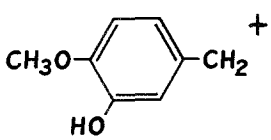
or more atoms of an isotope such as deuterium rather than the more abundant natural isotope. These fragments will be shifted on the mass scale by one or more atomic mass units and can be measured by other channels of the specific ion detector. Therefore, this method is essentially an isotope detection technique. The technique has been employed in the analysis of nortriptyline (5), prostaglandins (6) and amphetamines (7). Homovanillic acid (HVA) was derivatized to the heptafluorobutyryl methyl ester (8) and estimated with a labelled derivative (O-$^{2}H_3$-methyl) as an internal standard.

The position of the label in these compounds is important and is decided upon the basis of the fragmentation mechanism, for the label must be retained in an ion of relatively high abundance in order to obtain maximum sensitivity. A second requirement for an ideal internal standard is high isotopic purity.

We now report a simplified method for the determination of HVA, and 3-methoxytyramine (3-O-methyl dopamine) in which the isomeric compounds, iso-HVA and 4-O-methyl dopamine, serve as internal standards.

MATERIALS AND METHODS

HVA, 3-methoxytyramine, 4-methoxy-3-OH phenethylamine and 4-methoxymandelic acid (MMA) were purchased from commercial sources. Iso-HVA was obtained from Hoffmann-La Roche through the kind courtesy of Dr. Bouhon. A 4 N solution of ethanolic hydrochloric acid was prepared by passing hydrogen chloride gas through absolute ethanol. Freshly distilled Fisher reagent grade carbon disulfide was used. The ethyl acetate used in all extraction steps was of nanograde quality.

The preparation of the ethyl esters of HVA and iso-HVA, and the isothiocyanate (NCS) derivatives of 3- and 4-O-methyldopamine has been previously described (9,10). These compounds are distinctly separated on a 3.0% OV-225 column and yield identical mass spectra characterized by the base peak at *m/e* 137 due to  or its isomer 

Preparation of biological samples.

Urine. The preparation of the acid esters from urine samples has also been described (9).

Cerebrospinal fluid. Two 1-ml aliquots of lyophilized CSF were heated with ethanolic HCl for 2 hr. The solution was separated from the solid residue by centrifugation and the solvent was removed by evaporation under vacuum. The residue was then extracted into 2 ml of ethyl acetate and the ethyl acetate was removed by evaporation under vacuum. The acid esters thus obtained wwere redissolved in 20 µl of ethyl acetate and 2-µl aliquots of the solutions were used for GC-MS.

Serum/plasma HVA. One hundred mg of either lyophilized serum or plasma was homogenized with 2 ml of ethanolic HCl and the mixture was heated in a stoppered tube for 2 hr and then centrifuged. The supernatant was decanted and saved and the residue was washed with ethanol. The supernatant and washings were combined and evaporated to dryness under vacuum. The residue was extracted into ethyl acetate, centrifuged and the solution evaporated again. The final residue was then redissolved in 20 µl of ethyl acetate and 2-µl aliquots were used for GC-MS. The ethyl acetate insoluble material contained the amine fraction as hydrochloride from which amines were extracted into ethyl acetate after adjusting the pH to >9 with 2 drops of ammonia. The ethyl acetate solution was shaken with CS_2 for 30 min and the NCS derivative thus obtained was evaporated to dryness and redissolved in 20 µl of ethyl acetate. An aliquot of 1 or 2 µl was used for GC-MS.

Preparative TLC. In several biological samples, such as urine or plasma, when the total acid fraction was esterified and used directly for GC or GC-MS, it sometimes took 2 hr for it to elute completely from the GC column, then the mass spectrum was tied up and the output in analysis reduced. For this reason it was necessary to fractionate the ester fraction by preparative thin-layer chromatography (TLC). Chloroform:acetic acid (100:3) was found to be a satisfactory solvent system for the separation. The R_F value for HVA and iso-HVA ethyl ester was found to be 0.25. In all the chromatograms, standard HVA ester

Table 1. Relationship between quantity of HVA and iso-HVA injected and peak height

HVA		Iso-HVA		Ratio[a]
Quantity (µg)	Peak height (mm)	Quantity (µg)	Peak height (mm)	
0.11	74	0.10	47	0.70
0.11	90	0.10	59	0.72
0.20	180	0.02	13	0.72
0.11	21	0.10	13	0.68
0.10	43	0.10	31	0.72

[a]For 0.1 µg each of HVA and iso-HVA; the mean, SD and SEM for 20 determinations were, respectively, 0.703, ± 0.0177 and ± 0.005.

was spotted in 1- and 10-µg aliquots for recovery studies and as a reference, and the standard (1 µg) only was visualized by spraying with diazotized *o*-tolidine. The area corresponding to HVA and iso-HVA was eluted and analyzed by GC and GC-MS. In all cases standards were run simultaneously for recovery studies.

Trimethylsilyl (TMS) ethers. The TMS ethers of hydroxy compounds were obtained by heating the ester with bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane (Regisil^{R}) for 15 min at 60°C in a stoppered tube. The standard solutions were made up to a concentration of 0.05 µg/µl.

Selective ion monitoring

One µl of a standard containing a mixture of 0.1 µg of the ethyl esters of both HVA and iso-HVA was injected on the GC column and the mass spectrometer was focused on *m/e* 137. The tracings of the monitor for the GC peaks of both metabolites were recorded on oscillographic paper. The standards were run in duplicate four times with 2-hr intervals between the runs. Similarly, standard mixtures in varying proportions from 0.01 µg to 0.1 µg of iso-HVA with 0.1 µg HVA and from 0.01 to 0.1 µg of HVA with 0.01 µg of iso-HVA were run on SIM.

Table 2. Comparative data obtained by GC-FID[a] and GC-MS SIM[b] of the acidic fraction from human urine samples[c]

GC-FID		GC-MS SIM	
HVA (µg)	Iso-HVA	HVA (µg)	Iso-HVA (µg)
27.5	ND[d]	25.0	0.7
28.0	ND	30.0	1.2
ND	ND	0.01	ND
ND	ND	2.2	ND

[a]Gas chromatography-flame ionization detection.

[b]Gas chromatography-mass spectrometry single ion monitoring.

[c]The values represent the total quantity in a 0.5-ml aliquot of a bicarbonate extract of urinary acids.

[d]Not detectable.

One- or 2-µl aliquots of the sample out of 20, 50 or 100 µl, depending on the level in the sample, were injected both alone and in a mixture with standard iso-HVA (0.1 µg), which served as the internal standard. Similarly 4-O-methyl-dopamine NCS served as the internal standard for the determination of 3-O-methyl-dopamine NCS.

GC-MS analysis

A Varian CH-7 mass spectrometer was interfaced with a Varian 2740 gas chromatograph. The column conditions were: 6-ft, 3% OV-225 column, isothermal 190°C, helium flow-rate 30 ml/min, temperature of the Watson-Biemann separator and the ion source 300°C, ionization potential 70eV.

RESULTS

Under these GC column conditions HVA and iso-HVA are distinctly separated with retention times of 3 min 20 sec and 3 min 55 sec, respectively. Both isomers give the most abundant fragment ion at *m/e* 137. The isomeric compounds have identical GC-MS properties including the elution on the column and the yield

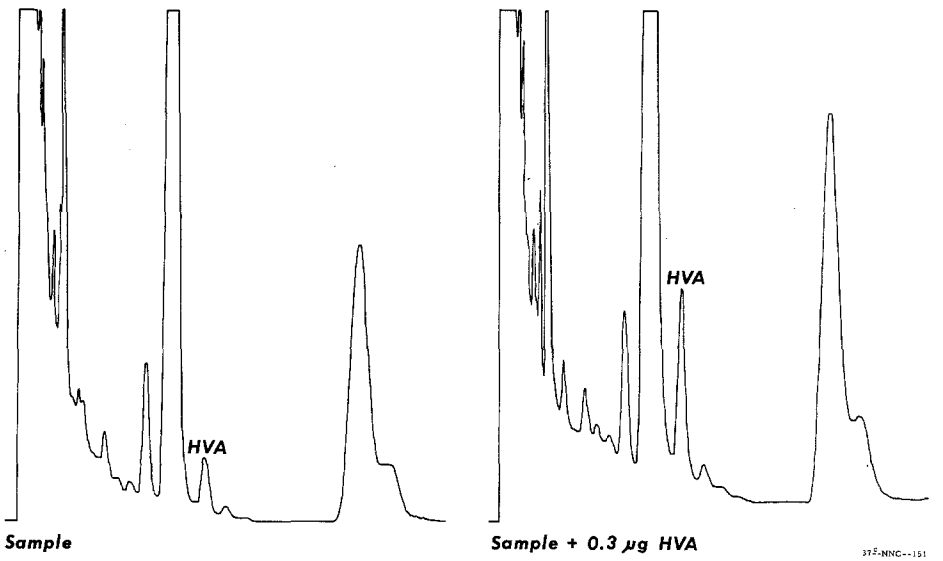
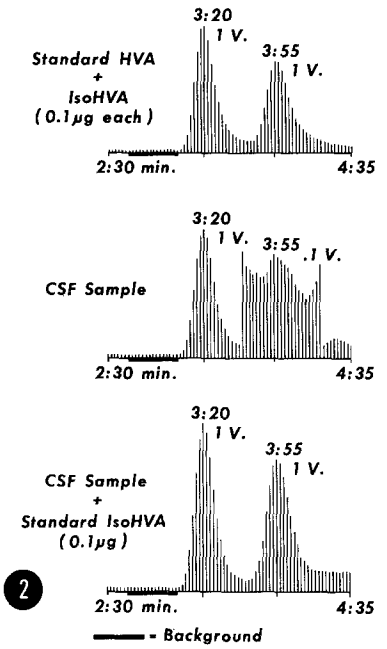


Fig. 1 Gas chromatographic chart of acid ethyl esters from cerebrospinal fluid of dog on L-DOPA (3% OV-225 on GCQ, 6-ft column, 160°C).

across the fritted glass separator. The peak heights for 0.1 µg of the standards were compared. Since iso-HVA has a longer retention time and elutes as a broader peak, the maximum height of the ion tracing is 70% of that of HVA. However, this ratio has been constant in the more than 100 determinations carried out at different sensitivity settings and with varying quantities injected. The methyl esters gave similar results with a ratio of iso-HVA/HVA of 0.5. A few values are presented in Table I. The GC chart of ethyl esters from CSF of dog on L-DOPA with and without added HVA is shown in Fig. 1.

Typical tracings of the SIM of a mixture of the ethyl esters of HVA and iso-HVA, of a sample of CSF from dog and of an internal standard of iso-HVA are shown in Fig. 2. In a series of determinations in which the single ion was monitored, the level of iso-HVA was never more than 5% of that of HVA. Since the background contribution of endogenous iso-HVA to the peak height of the internal standard is negligible, the concentration of HVA is calculated directly on the basis of the peak height of the internal standard. For example, tracings of *m/e* 137 for 0.1 µg of iso-HVA and HVA give peak heights of 42 mm and 60 mm, respectively. Thus the level of HVA in a sample is calculated from the actual



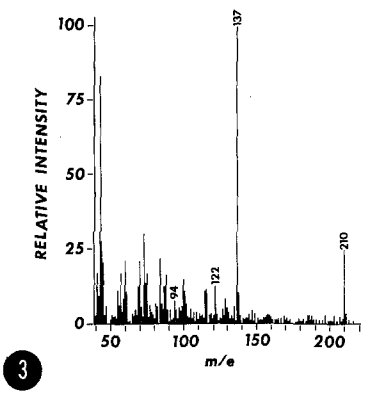


Fig. 2 SIM tracings of the ion at *m/e* 137: standard mixture of HVA and iso-HVA (upper), cerebrospinal fluid sample (center) and cerebrospinal fluid sample plus internal standard (lower). The values of HVA calculated from sample alone (center) and from samples and internal standard (lower) are 1.0 μg/ml and 0.97 μg/ml, respectively.

Fig. 3 Mass spectrum of the GC peak of HVA ethyl ester from a lyophilized sample of cerebrospinal fluid from dog.

peak height for HVA in the sample and for iso-HVA in the internal standard. The reproduciblity of the results obtained by this method was confirmed by replicate analyses, by analyses of the same sample on different days when the sensitivity of the instrument varied and by comparison with the results obtained by GC with the flame ionization detector. The results are summarized in Table 2. In a few typical cases the results were further confirmed by running the TMS ethers of the ethyl ester and monitoring the base peak at *m/e* 209 for the quantitation. When the level of HVA was less than 1 mg in a 24-hr urine sample, only SIM gave reliable results.

Table 3. Levels of HVA and iso-HVA in cerebrospinal fluid of humans

HVA (μg/ml)	Iso-HVA (μg/ml)	Iso-HVA / HVA (%)	HVA + iso-HVA (TMS) (μg/ml)
0.30	0.012	4.0	0.35
0.12	0.005	4.2	0.15
0.40	0.013	3.2	----
0.025	ND[a]		----
0.06	ND		0.05

[a]Not detectable.

Table 4. Levels of HVA and iso-HVA in cerebrospinal fluid of dogs given L-DOPA (1 g/day for 42 days)

HVA (μg/ml)	Iso-HVA (μg/ml)	Iso-HVA / HVA (%)
1.80	0.092	5.0
1.52	0.066	4.4
0.90	0.04	4.4
1.20	0.044	3.7

The levels of HVA and iso-HVA in the CSF of four human subjects with different neurological diagnoses were determined by this method (Table 3). In quantitating HVA and iso-HVA in CSF samples from the human and dog, the identity of the peak being estimated was established by its mass spectrum. A typical spectrum obtained with a sample of CSF from dog is shown in Fig. 3. The levels of HVA and iso-HVA in CSF of dogs after the chronic administration of L-DOPA were similarly determined (Table 4).

MMA TMS ether methyl ester and HVA TMS ether ethyl ester are well separated (retention times 3 min 28 sec and 4 min 50 sec, respectively) on a 6-ft, 3% OV-

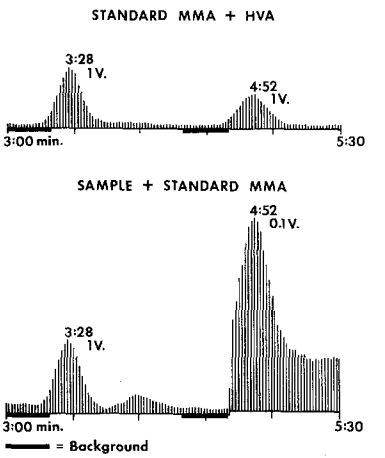


Fig. 4 SIM tracings: standard mixture of 0.025 μg each of MMA TMS ether methyl ester and HVA TMS ether ethyl ester (upper), TMS ether ethyl ester of cerebrospinal fluid sample (lower). The value for HVA obtained from the peak height in the sample is 11.6 ng.

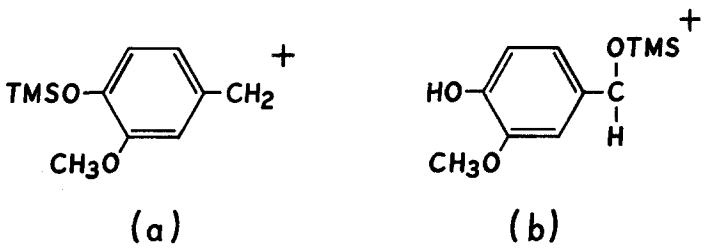
225 column at 170°C isothermal and yield a constant ratio of response for the base peak at *m/e* 209. Typical tracings of the SIM at *m/e* 209 for a mixture of 0.025 μg of each of the two acids and for a sample with added internal standard are shown in Fig. 4.

DISCUSSION

The methods for the determination of HVA previously reported in the literature have been elaborate and complicated (8,11) and have not taken into account the presence of iso-HVA. In the method described here, the procedures for the preparation of ethyl ester, the separation of the isomeric compounds and their quantitation by SIM are relatively simple. The isomeric compound with identical fragmentation, the same relative abundance of fragment ions and the same GC characteristics is an ideal internal standard and is, in addition, less expensive than isotopically labelled compound. In a series of communications we have reported that the OV-225 GC column is the most suitable for the separation of isomeric compounds. In our experience, esterfication of lyophilized CSF is the

most suitable method, for at the low concentrations usually present in CSF and plasma, recovery by the extraction method is poor and inconsistent. Furthermore, the sample can be further purified by preparative TLC when a major compound interferes on GC-MS. However, in samples of CSF from humans and dogs no interference by any other compound was noted. The mass spectra of HVA in CSF samples from humans and dogs were identical to the spectrum of standard HVA and showed no contamination. In HVA there was no background noise for the peak at *m/e* 137, and in iso-HVA, since the sensitivity setting is higher, the background noise is subtracted from the peak height. After the determination of HVA and iso-HVA in the sample by two injections, one with and one without an internal standard, the remaining 80% of the ester fraction was derivatized to the TMS ether. The TMS ether ethyl esters of HVA and iso-HVA, which are not separated, are assayed together by monitoring the base peak at *m/e* 209.

MMA and HVA are also isomeric compounds. The mass spectra of both are characterized by the base peak at *m/e* 209 due to fragment ions (a) and (b).



MMA TMS ether ethyl ester and HVA TMS ether ethyl ester do separate on a 3% OV-225 column at 170°C isothermal (retention times 3 min 55 sec and 4 min 50 sec, respectively). Since the separation is even better with MMA TMS ether methyl ester (retention time 3 min 28 sec), the methyl ester was used as the internal standard for the determination of HVA and iso-HVA together. This procedure was used as a second method to confirm the levels of HVA and iso-HVA and also, when necessary, to eliminate any ambiguity due to interfering GC peaks

A ratio of iso-HVA to HVA in the range of 2-4.5% was found in CSF from five

human subjects and ten dogs and in the blood and urine of human subjects. The ratio remained unchanged in the CSF of humans and dogs after the administration of L-DOPA. Full details of clinical reports and detailed analysis of other metabolites of L-DOPA will be subject matter of a separate communication.

This method, which is simpler and equally as sensitive as those reported in the literature, has the added advantage of selectivity for HVA and iso-HVA. In our experience even under the mildest of the conditions used (1 min at 0°C), with ethereal diazomethane, dihydroxyphenylacetic acid (DOPAC) gave 3 to 5% of HVA methyl ester and 0 to 1% of iso-HVA methyl ester. Since in biological samples, such as plasma and CSF, there was evidence of DOPAC (unpublished work) we preferred the alcoholic HCl method for esterification.

ACKNOWLEDGEMENTS

We are grateful to Dr. Bouhon who shared with us his gift of iso-homovanillic acid from Hoffmann-LaRoche Inc. The author thanks John Bogulski, Cynthia Brown and Katharine Leiner for their excellent technical assistance in GC-MS, TLC and GC analysis, respectively.

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