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MEASUREMENT OF PLASMA VANILLYLMANDELIC ACID BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A liquid chromatographic method with electrochemical detection is described for measuring plasma 3-methoxy-4-hydroxymandelic acid (VMA). Plasma is deproteinized by gel filtration and VMA is extracted into ethyl acetate, which is evaporated. VMA is oxidized to vanillin, which is purified by toluene extraction and quantified by high-performance liquid chromatography. The recovery of VMA through the entire procedure is 52 ± 10% (mean ± S.D., n = 19). The plasma VMA concentration in healthy young volunteers varies between 4.39 and 14.6 ng/ml, a range that is in excellent agreement with data obtained with mass fragmentography.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been widely utilized to measure catecholamine metabolites in plasma and urine. Of the major metabolites, 3-methoxy-4-hydroxymandelic acid (VMA) has been the most difficult to measure with HPLC, because its high polarity results in poor retention in most reversed-phase systems [1, 2] and its elution near the solvent front is often obscured by multiple interfering peaks [3, 4]. Although many HPLC methods for measuring VMA in urine have been described, we are aware of only one previous HPLC method for measuring plasma VMA [5]. Since we questioned the specificity of this method, our approach has been to oxidize the VMA to vanillin, a non-polar product, which can readily be separated from interfering compounds.

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EXPERIMENTAL

Reagents

All chemicals were reagent grade and purchased from Fisher Scientific (King of Prussia, PA, U.S.A.). HPLC-grade methanol and ethyl acetate were also purchased from Fisher Scientific. The internal standards VMA and vanillin were purchased from Sigma (St. Louis, MO, U.S.A.). 2-[^{14}C]VMA was purchased from Research Products International (Mount Prospect, IL, U.S.A.) and Bio-Gel P-10 resin (100—200 mesh) was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Vitamin B_{12} (1 mg/ml) was purchased from Elkins-Sinn (Cherry Hill, NJ, U.S.A.).

Gel chromatography column

The Bio-Gel P-10 column was poured into a 30-ml disposable syringe with an eluting buffer of 0.05 *M* sodium phosphate (pH 6.0). The final bed volume was 20 ml. Vitamin B_{12} (mol. wt. 1350) was used as a marker to determine the elution volume of VMA. When added to plasma, its distinctive red color indicates the elution of low-molecular-weight species. Using 2-[^{14}C]VMA, we established that VMA eluted immediately after vitamin B_{12}.

Sample preparation

Blood was collected in 5-ml tubes containing 100 μl of ethylene glycol tetraacetate (EGTA) (90 mg/ml) and glutathione (60 mg/ml). The tubes were immediately centrifuged at 1400 *g* for 10 min, and plasma was stored frozen at -15°C. On the day of analysis, each plasma sample was divided into three identical 1.5-ml aliquots, one serving as a sample, one as a blank and the third was spiked with 40 ng of VMA. After vitamin B_{12} (100 μg) was added, each aliquot was applied to a separate Bio-Gel P-10 column. Eluting buffer was added manually with a Pasteur pipet and 15 ml of eluate were collected, beginning with the appearance of vitamin B_{12}. Each eluate was divided in two (so duplicate determinations could be made on all three aliquots) and poured into 50-ml screw-top tubes. Potentially interfering non-polar compounds (dietary vanillin, MPHG) were extracted into ethyl acetate by shaking gently on an Eberbach shaker (Arthur Thomas, Philadelphia, PA, U.S.A.) for 5 min. Following centrifugation for 2 min at 200 *g*, the organic phase was discarded. The aqueous phase was adjusted to pH 3.0 with concentrated phosphoric acid, 2 g of sodium chloride were added, and the VMA was extracted twice into 20 ml of ethyl acetate by shaking gently for 5 min as described above. The organic phases were pooled in 50-ml Pyrex tubes and evaporated to dryness under nitrogen in a 37°C water bath. The dry residue was stored frozen overnight.

Oxidation

The residue was reconstituted by adding 200 μl of 0.5 *M* ammonium hydroxide to each sample, vortexing briefly, and centrifuging for 2 min at 200 *g*. The ammonium hydroxide was then transferred to 100 mm × 13 mm I.D. borosilicate glass test-tubes and allowed to stand for 20 min at room temperature before the oxidation was initiated by the addition of 20 μl of 4% sodium metaperiodate. The samples and standards were placed in a water bath at

37°C for 5 min, and then the reaction was stopped by placing the tubes in an ice bath and adding 25 μl of glacial acetic acid. Sodium metaperiodate was not added to the non-oxidized blanks, which were kept in the ice bath during the incubation, before neutralization with 25 μl of glacial acetic acid.

The vanillin was extracted into 600 μl of toluene by vortexing for 1 min, then centrifuging for 2 min at 200 *g*. The aqueous phase was frozen for 12 s in a dry ice–methanol bath and the toluene decanted into another test-tube. The vanillin was back-extracted into 200 μl of 1.0 *M* ammonium hydroxide by once again vortexing for 1 min and centrifuging for 2 min at 200 *g*. The aqueous phase was frozen and the organic phase was aspirated and discarded. A 150-μl aliquot of the aqueous phase was neutralized with 30 μl of glacial acetic acid.

High-performance liquid chromatography

The HPLC system consisted of a Model 6000A pump, a Model 680 gradient controller, a U6K injector and a 5-μm Novapak C_{18} (15 cm × 4.6 mm I.D.) reversed-phase column, all from Waters Assoc. (Milford, MA, U.S.A.). The electrochemical detector was a Model LC-4B with a glassy carbon working electrode and an Ag/AgCl reference electrode from Bioanalytical Systems (West Lafayette, IN, U.S.A.). The mobile phase consisted of a mixture of 0.07 *M* potassium phosphate (pH 7.0)—methanol (80:20), filtered through a 0.45-μm Nylon-66 membrane and degassed under vacuum. The flow-rate was 1.0 ml/min and the detector potential was set at +0.85 V versus the Ag/AgCl chloride reference electrode.

Calculations

Peak height was measured directly from a chart recorder. Plasma VMA concentration was determined by the formula:

$$VMA_{sample} = \frac{PH_s - PH_b}{PH_{std} - PH_s} \times VMA_{standard\ added}$$

where PH_b is the peak height of non-oxidized blank, PH_s is the peak height of sample, and PH_{std} is the peak height of standard.

Assessment of recovery, linearity, specificity and reproducibility

The recovery of VMA through the whole procedure was calculated as follows:

$$\text{Percentage recovery} = \frac{(\text{vanillin in spiked sample} - \text{vanillin in sample}) \times 1.3}{20\ \text{ng}}$$

The correction factor of 1.3 accounts for the molecular weight difference between VMA and vanillin.

To establish that the linearity of the method is maintained even in states of catecholamine excess, we added standard VMA in concentrations (0, 10, 25, 50 and 100 ng/ml) that extended well above the range we anticipated in healthy controls. The response (peak height) resulting from the added VMA was calculated by subtracting that resulting from endogenous VMA in the plasma sample.

The specificity of the method was assessed by adding 25 ng of a series of structurally related compounds at the beginning of the procedure, including the known catecholamine metabolites 3-methoxy-4-hydroxyphenylacetic acid (HVA) and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG). The compounds tested had the 3-methoxy-4-hydroxy nucleus and were potential substrates for vanillin formation.

Inter-assay coefficient of variation (C.V.) was determined by measuring plasma VMA on seven separate occasions on pooled plasma (35 ml total) from three normal volunteers; the VMA concentration in the pooled sample was 10.1 ng/ml.

RESULTS

Vanillin can easily be resolved and quantified with the present HPLC method (Fig. 1). The identity of the oxidation product was verified with a hydrodynamic voltammogram; the profile of the vanillin formed by oxidizing VMA in the plasma extract (8.5 ng/ml) was identical to the profile of authentic vanillin (Fig. 2). Specificity was further evaluated by adding 25 ng of a series of related compounds (Table I) at the beginning of the entire procedure. Only one of the compounds, 3-methoxy-4-hydroxyphenylpyruvic acid (vanillylpyruvic acid, VPA), cross-reacted with VMA in this assay. VPA, a minor metabolite of dihydroxyphenylalanine, if actually present in plasma, could result in significant interference. For this reason, we allowed the samples to stand in ammonium hydroxide for 20 min prior to performing the oxidation. Under these conditions, VPA spontaneously degrades [6] and no interference is observed.

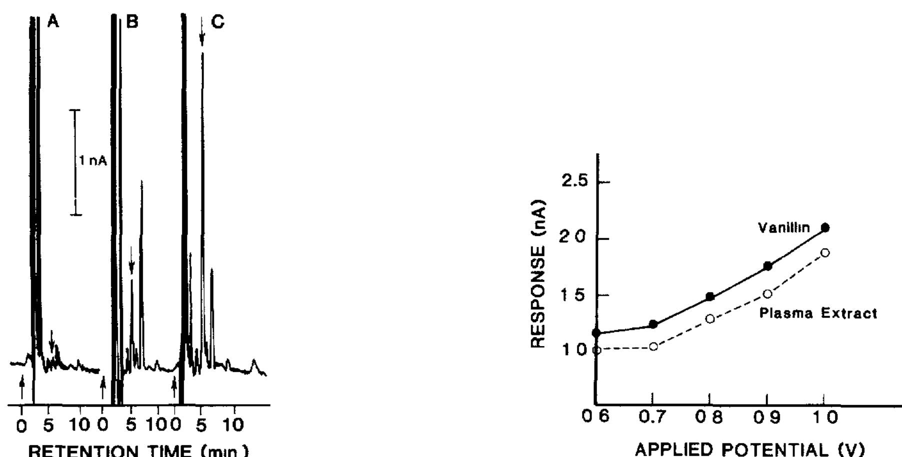

Fig. 1. Chromatogram of a normal plasma extract processed as explained in the text. (A) Non-oxidized blank, 50 µl injected; (B) sample, 50 µl injected; (C) sample spiked with VMA, 25 µl injected. The arrows pointing upwards indicate the time of the injections; the arrows pointing downwards indicate the elution of vanillin.

Fig. 2. Hydrodynamic voltammogram of authentic vanillin and vanillin formed by oxidation of VMA in a plasma extract The concentration of VMA in the plasma extract was 8.5 ng/ml.

TABLE I

COMPOUNDS TESTED FOR INTERFERENCE

Normetanephrine	3-Methoxy-4-hydroxyphenylpyruvic acid** (VPA)
3-Methoxy-4-hydroxyphenylethyleneglycol (MHPG)	3-Methoxy-4-hydroxyphenyllactic acid
3-Methoxy-4-hydroxyphenylacetic acid* (HVA)	3-Methoxy-4-hydroxycinnamic acid (ferulic acid)
3-Methoxy-4-hydroxybenzylalcohol	3-Hydroxy-4-methoxymandelic acid (iso-VMA)
3-Methoxy-4-hydroxybenzoic acid	5-Hydroxy-2-indolecarboxylic acid

*Homovanillic acid.
**Vanillylpyruvic acid.

TABLE II

VMA CONCENTRATION IN RANDOM PLASMA SAMPLES FROM HEALTHY VOLUNTEERS

The subjects were sitting or standing prior to sample collection No dietary instructions were given.

Subject	VMA (ng/ml)
1	11.3
2	5.98
3	8.58
4	10.1
5	8.89
6	14.6
7	7.78
8	9.30
9	4.39

TABLE III

COMPARISON OF METHODS FOR MEASURING PLASMA VMA

	Reference	VMA (ng/ml)	Method	C V. (%)
This method		4.39—14.6*	HPLC	10.7
Ong et al.	5	4.27— 4.77	HPLC	—
Sjoquist	7	6.1 —14.1	GC—MS	13.4
Takahashi et al.	8	10.0 —20.0	GC—MS	—
Hjemdahl et al.	9	11.8 ± 1.9**	GC—MS	—
Hunneman	10	9.0 ± 4.0	GC—MS	11.0

*Range.
**Mean ± S.D.

A linear relationship between VMA added and vanillin formed was observed over the working range of VMA concentrations (10—90 ng). Least-squares regression analysis indicated no significant deviation from linearity (r = 0.9984). The equation of the best-fit line was $y = 0.11x - 0.33$. The detection limit, based on a signal-to-noise ratio of 2, was found to be 1.0 ng of VMA per ml of plasma. The routinely observed reading to blank ratios varied between 5 and 20.

The observed range for the plasma VMA concentration in healthy young volunteers was 4.39—14.6 ng/ml (Table II). This was in excellent agreement

with data obtained with mass fragmentographic methods [7—10] (Table III). The average recovery of VMA was 52.4 ± 10.9% S.D. (n = 19). The inter-assay C.V. was 10.7% (n = 7).

VMA was stable in frozen plasma for at least two months.

DISCUSSION

Catecholamine metabolites can readily be identified and quantified electrochemically in HPLC eluates. The potential for interference from dietary constituents and drug metabolites is high, however, and the specificity of many published methods has never been rigorously established. In a recent liquid chromatographic study, based on in-series coulometric electrodes, interfering compounds were observed to cross-react in standard HPLC methods for dopamine, dihydroxyphenylacetic acid and MHPG [11]. Because of the polar nature of the VMA molecule, it has a short retention time on reversed-phase HPLC columns, and coelution of interfering compounds is particularly worrisome. Nevertheless, Ong et al. [5] were able to identify and quantify VMA in HPLC eluates of plasma extracts. We were unable, however, to duplicate their results. Although we confirmed, using the described chromatographic conditions, the excellent retention of VMA (9 min), analysis of plasma extracts revealed multiple interfering peaks, making it difficult or impossible to identify VMA. MHPG, by contrast, was retained for 16 min in this system and was readily separated from the multiple compounds that eluted earlier.

Since we were unable to identify VMA in plasma extracts, we elected to oxidize the VMA to vanillin. This approach was attractive because it made it possible to exploit the great differences in polarity between VMA and vanillin and to ensure the specificity of our method. Several methods for urinary VMA are based on this principle [12, 13]. Washing the Bio-Gel P-10 column eluate with ethyl acetate at a pH of 6 removes not only dietary vanillin but also MHPG and many other non-polar compounds, which might interfere in the vanillin assay at the end of the procedure. Thus, following the first ethyl acetate extraction, only relatively polar molecules, like VMA, remain in the aqueous phase. We suspect that few of these polar constituents extract into toluene (a hydrophobic medium) following the oxidation. For these reasons, only two or three peaks, other than vanillin, are present in our final chromatograms (Fig. 1). Of the potentially interfering compounds tested, only VPA cross-reacted in this assay. This possible source of interference was eliminated by pre-incubating the samples in ammonium hydroxide prior to the oxidation (see Results). We view this step as a precaution, since we are unaware of data documenting the presence of VPA in normal human plasma or food.

MHPG also oxidizes to vanillin under the conditions described, but it does not interfere with this assay since it is removed by the first ethyl acetate extraction. It is not necessary to restrict dietary vanillin in patients studied with this method, since we established that vanillin also is removed in the first ethyl acetate extraction. Any dietary vanillin remaining in the aqueous phase following the ethyl acetate wash would not obscure our quantitation of VMA, since all our sample readings are corrected by subtracting the readings obtained with non-oxidized blanks (see *Calculations*).

Although the sample preparation is more extensive than in most HPLC methods, the oxidation to vanillin and toluene extraction adds only a few hours to the assay. The use of vitamin B_{12} as a marker for VMA elution from the Bio-Gel P-10 column has greatly expedited the gel filtration and allowed us to run several columns simultaneously. This makes it possible to analyse twelve to fifteen samples per week. We have not, however, found a suitable internal standard for this assay. Iso-VMA (4-methoxy-3-hydroxymandelic acid) is a possibility, since isovanillin can readily be separated from vanillin in our HPLC system. Unfortunately, iso-VMA is removed from the aqueous phase in the ethyl acetate wash. The inter-assay C.V. (10.7%) was satisfactory without an internal standard.

The method, hopefully, will be useful for measuring catecholamine production in vivo and particularly for diagnosing catecholamine-secreting tumors. We have established that the linearity of the method extends to at least 100 ng VMA per ml plasma, well above the range observed in our normal subjects. Using this method, we observed that the plasma VMA concentration in healthy young volunteers ranges between 4.39 and 14.6 ng/ml, which agrees well with mass fragmentographic data.

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