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Note

Determination of vanillylmandelic acid in plasma by high-performance liquid chromatography with electrochemical detection

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The availability of a method for monitoring norepinephrine (NE) metabolites in human tissues and body fluids could be useful in preclinical and clinical neuropharmacology to assess noradrenergic system activity.

It has been reported that depressive disorders [1] and stress states [2] might be due to a dysfunction of the central noradrenergic system. Urinary excretion of vanillylmandelic acid (VMA), the main acidic metabolite of NE, has been used in the diagnosis of catecholamine-secreting tumours [3] and to monitor the turnover of NE in the sympathetic nervous system (SNS) [4]. In addition, recent evidence of complex interactions between the SNS and the central nervous system (CNS) [5] suggests that measurement of VMA in cerebrospinal fluid (CSF) [6] and plasma can give indications on the central NE turnover in humans. The use of high-efficiency reversed-phase high-performance liquid chromatography (HPLC) columns coupled with specific and very sensitive electrochemical detectors permits the determination of very small amounts of catecholamine metabolites in biological samples. Here we report a rapid and sensitive HPLC method using a dual coulometric detector for the quantitation of VMA in human plasma.

EXPERIMENTAL

Chemicals

VMA was purchased from Sigma (St. Louis, MO, U.S.A.) and isovanillylmandelic acid (iso-VMA) from Fluka (Buchs, Switzerland). All the other reagents were of the best analytical grade and were obtained from common commercial sources. Concentrated solutions of standards were prepared in redistilled deionized water and kept frozen at $-20\,^{\circ}C$ until use.

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Chromatographic system

The chromatograph consisted of a Knauer 64 HPLC pump (Bad Homburg, F.R.G.), a Rheodyne (Cotati, CA, U.S.A.) Model 7125 injection valve with a 100-μl loop, a Waters (Milford, MA, U.S.A.) Guard Pak precolumn packed with 5 μm particle size C_{18} material, a 250 mm $\times$ 4.6 mm I.D. Baker (Deventer, The Netherlands) C_{18} (5 μm particle size) analytical column and an ESA (Bedford, MA, U.S.A.) Model 5100A coulometric detector with a Model 5011 analytical cell and a Model 5021 conditioning cell. The potentials were +0.20 V for the conditioning cell and +0.40 V for the first electrode of the analytical cell; the second electrode was not used.

The signal from the analytical cell was recorded on a Houston Instruments (Gistel, Belgium) Omniscribe recorder. The detector gain was set to 8×100 with a full-scale sensitivity of 0.125 μA.

The mobile phase was 0.04 *M* sodium dihydrogenphosphate and 0.005 *M* sodium citrate; the pH was adjusted to 3.4 ± 0.1 and the solution was filtered through a 0.22-μm Millipore filter and degassed under reduced pressure. All analyses were carried out isocratically at room temperature with a flow-rate of 1.0 ml/min.

Sample handling

Plasma samples (2 ml) obtained from heparinized blood were transferred to plastic tubes each containing 100 μl of iso-VMA solution (0.13 ng/μl). After vigorous mixing and addition of 200 μl of 4 *M* perchloric acid the samples were centrifuged at 30 000 *g* for 25 min at 4°C and the supernatant was extracted with two 2-ml portions of ethyl acetate.

To facilitate the separation between the phases, the tubes were placed for 3 min in ethylene glycol at −25°C. The pooled organic phase was back-extracted with two 1-ml portions of 1 *M* sodium hydrogencarbonate. After washing with 4 ml of diethyl ether, the aqueous phase was brought to pH 3.4 by adding 1 ml of 1 *M* citric acid buffer (pH 1). Finally the solution was extracted with two 2-ml portions of ethyl acetate and the organic phase was pooled and evaporated to dryness under reduced pressure. The residue was redissolved in 250 μl of mobile phase and 100 μl were injected into the chromatograph.

Free plasma 3-methoxy-4-hydroxyphenylglycol (MHPG) was estimated with a method previously described [7]. Every sample was analysed in duplicate and the mean values were calculated.

Calculations

A plasma pool was obtained from twelve subjects of the laboratory staff. A standard curve was constructed by adding to each sample increasing amounts of VMA (1, 2, 4, 8, 16 and 32 ng) and a fixed amount of iso-VMA as internal standard (100 μl of a 0.13 ng/μl solution). The concentration of VMA in a given sample was calculated according to the following equation:

$$\text{VMA (ng/ml)} = \frac{\text{peak height of VMA}}{\text{peak height of iso-VMA}} \times \frac{1}{\text{slope}}$$

RESULTS

Chromatograms

Typical chromatograms are illustrated in Fig. 1. Fig. 1A represents an injection of an authentic aqueous solution of VMA (1 ng) and iso-VMA (1 ng). Fig. 1B and C are plasma samples without and with the addition of iso-VMA as internal standard. The retention times for VMA and iso-VMA are 7.5 and 12.1 min, respectively. Samples can be injected at intervals of ca. 20 min.

Peak identity

The purity of the VMA peak in plasma samples was checked using three methods. When the ionic strength, the pH or the organic eluent percentage was changed, the retention time of the VMA peak in the standards and in the plasma samples changed in an identical manner. In the second approach, plasma samples were spiked with a known amount of pure VMA standard; the peak shape did not change and the peak heights were proportional to the dose added (data not shown). Finally, the response to different voltage settings was compared for the VMA peak in standard and plasma samples. Also in this case, the strict similarity between the two chromatograms shows that no substance coelutes with VMA.

Linearity and calibration curve

The detector response for VMA was linear within a concentration range from 0.1 to 10 ng per injection. This range covers the values found in our samples. The

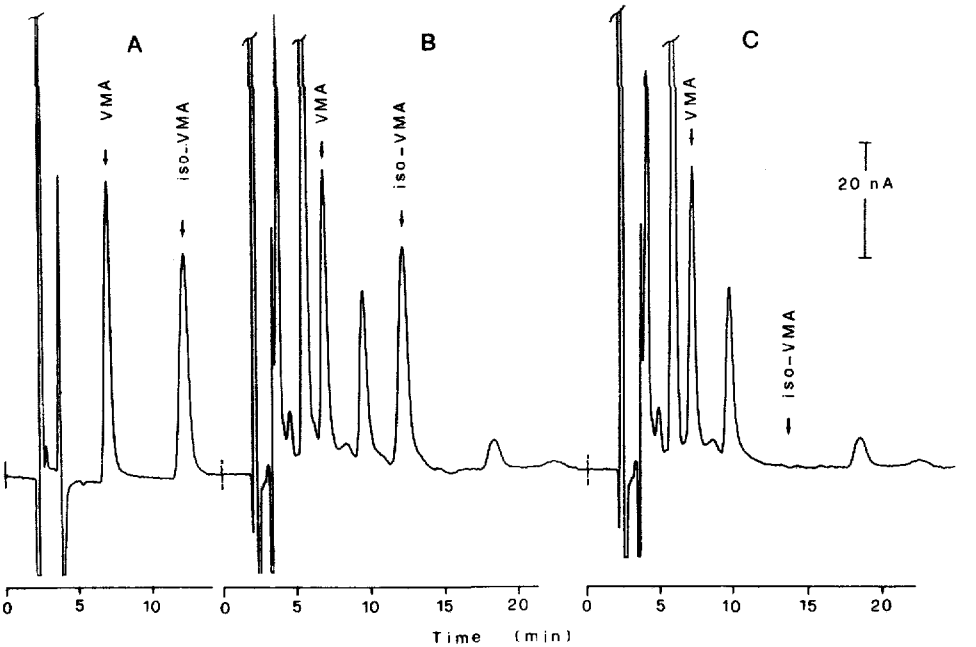

Fig. 1. Typical chromatograms of (A) VMA and iso-VMA standards (1 ng each), (B) plasma sample with 4.21 ng/ml iso-VMA added and (C) human plasma sample. For details, see text.

calibration curve yielded a regression line whose equation is $y=0.136x+1.073$ ($r=0.996$). The slope value was used in the formula described in the calculation section.

Detection limit and precision

Assuming a signal-to-noise ratio of 3, we can determine as little as 0.38 ng/ml VMA in human plasma. Intra-assay ($n=10$) and inter-assay ($n=10$) coefficients of variations at mean ($\pm$S.D.) concentrations of VMA of 9.05 ± 0.17 and 6.51 ± 0.21 ng/ml were 5.4 and 9.2%, respectively.

Plasma values

The VMA and free MHPG plasma concentrations in twelve apparently healthy human subjects (six males and six females) aged 43.3 ± 12.7 years (mean $\pm$ S.D.) (range 25–60 years) were 7.15 ± 3.05 ng/ml (mean $\pm$ S.D.) for VMA and 3.67 ± 0.86 ng/ml (mean $\pm$ S.D.) for free plasma MHPG.

These values agree well with the literature data [8,9]. When free plasma MHPG and plasma VMA were matched, a statistically significant ($P<0.001$) high correlation coefficient ($r=0.933$) was obtained.

DISCUSSION

In the literature most of reported methods describe the detection of VMA in urine, where relatively large amounts of this acidic metabolite are present [10–12]. Major problems arise when one deals with CSF or human plasma, and hence the few studies for VMA dosage in these two biological fluids use gas chromatography–mass spectrometry (GC–MS) [8,9]. To develop our method we had to focus our efforts on the clean-up procedure and on the relative specificity of the dual coulometric detector. A three-step extraction purification was necessary owing to the complexity of the biological matrix; in fact when in our experiments we tried to omit any extraction step, we obtained a chromatogram with peaks interfering with the two analytes (VMA and iso-VMA). Washing with diethyl ether and the subsequent extraction at pH 3.4 led to a chromatographic tracing free from impurities. The clean-up procedure, yielding low recoveries (32.6 ± 2.9% for VMA, mean $\pm$ S.D. of ten experiments at a concentration of 10 ng/ml of plasma), forced us to look for a reliable internal standard.

The choice of iso-VMA was prompted by previous reports in the literature [13,14]. Although one paper reported endogenous levels of iso-VMA [15], during our experiments we had no problems. The availability of a dual coulometric detector allowed us to screen the most suitable combination of oxidation potential.

The first operating electrode in the conditioning cell is set at +0.20 V and oxidizes the low-potential compounds and impurities in the mobile phase; at this potential VMA does not undergo any appreciable electrochemical transformation. This situation results in a better signal-to-noise ratio with a stable baseline. The method is sensitive enough to detect VMA levels in 1 ml of human plasma; as to speed, a skillful technician can process ten plasma samples, ready for injection into the chromatograph, in less than 1 h. Recent studies employing the direct

injection into humans of labelled compounds demonstrated that a substantial portion of free plasma MHPG is rapidly converted into VMA [16,17]. A strict relationship between these NE metabolites is also supported by the statistically significant positive correlation we found in our samples, in agreement with a previous report [18]. These considerations suggest that the measurement of VMA in human plasma can give a more comprehensive index in NE turnover in humans [19].

Indeed, the activity of the enzymes converting MHPG into VMA may be influenced by some factors such as diet, drugs and stress [16]; moreover, plasma VMA is formed in tissues not only from free MHPG but also probably from other neutral catecholamine metabolites [16]. Finally, the reliability of urinary evaluation of these metabolites could be weakened by changes in renal clearance and/or renal metabolism [18]. For these reasons, we propose that plasma VMA measurements can give further information when short-term changes in NE metabolism have to be monitored, as in studies on the effect of physical activity [20] and stressful situations [21].

In conclusion, we believe that this method represents a valid alternative to methods employing expensive GC–MS equipment.

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