*Journal of Chromatography*, 433 (1988) 23–30
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4436

# EFFECTS OF LONG-TERM STORAGE ON THE CONCENTRATIONS OF THE UNCONJUGATED ACIDIC METABOLITES OF THE TRACE AMINES, INDOLEAMINES AND CATECHOLAMINES

B.A. DAVIS

*Neuropsychiatric Research Unit, Medical Research Building, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0 (Canada)*

(First received June 22nd, 1988; revised manuscript received August 8th, 1988)

## SUMMARY

The concentrations of urinary phenylacetic, *m*- and *p*-hydroxyphenylacetic, mandelic (MA), *p*-hydroxymandelic, indoleacetic, homovanillic, vanillylmandelic, 5-hydroxyindoleacetic and 3,4-dihydroxyphenylacetic (DOPAC) acids and 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) and the concentrations of the above plasma acids (except MA, MHPG and DOPAC) were determined by gas chromatography–mass spectrometry with selected-ion monitoring at the time of receipt of the samples and after three, six and nine months storage at −18°C. The samples were not treated in any way before storage. The concentrations of most of the urinary metabolites declined significantly (analysis of variance) over the nine-month storage period, whereas most of the plasma metabolites did not.

## INTRODUCTION

It is frequently neither possible nor practical to carry out quantitative determinations on biological samples on the same day that they are received in the laboratory. Storage of the samples for quite long periods of time may be necessary. During storage some of the analytes of interest may decompose, the rate of decomposition depending on temperature, pH, the presence of light, enzymes or other factors. When finally analyzed, the observed concentrations will be lower than the true values. The losses due to decomposition can be automatically corrected by the use of deuterium-labelled analogues as internal standards and mass spectrometry (MS) for quantification. However, for very labile substances and very long storage times, both the deuterio and protio compounds may decompose. It is of interest, therefore, to ascertain the extent of decomposition of the major metabolites of the biogenic amines in biological materials after long periods of

0378-4347/88/$03.50 

storage. This information is essential no matter what method of quantification is used.

The stabilities of 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG) [1–5], 5-hydroxyindoleacetic acid (5-HIAA) [6–9], homovanillic acid (HVA) [8,9] and vanillylmandelic acid (VMA) [4] have been investigated by a number of authors, mostly for relatively short storage periods of a month or less and mostly for urinary or standard solutions. The effects of longer periods of storage on these metabolites and on the "trace" acids have not been investigated. In this study, eleven urinary and eight plasma metabolites have been quantified by capillary column gas chromatography–high-resolution mass spectrometry (GC–MS) with selected-ion monitoring (SIM) after three, six and nine months of storage of the untreated samples.

## EXPERIMENTAL

### *Chemicals and reagents*

All organic solvents were glass-distilled HPLC grade (Caledon, Georgetown, Canada). Pentafluoropropionic anhydride was purchased from Pierce (Chromatographic Specialties, Brookville, Canada) and trifluoroethanol from Aldrich (Milwaukee, WI, U.S.A.). The deuterium-labelled acids used as internal standards were synthesized as described previously [10] except for [$^2H_3$]MHPG piperazine salt which was obtained from Merck Sharp and Dohme (Montreal, Canada). The abbreviations used for the metabolites are explained in Table I.

### *Instrumentation*

GC–MS analysis was performed with a VG 70-70F double-focussing mass spectrometer equipped with an HP 5700 gas chromatograph. The outlet of a J & W Scientific bonded-phase silica column, DB-1, 60 m$\times$0.32 mm I.D., was inserted directly into the ion source. For SIM, the mass spectrometer was operated at a resolution of 5000. The operating conditions for the gas chromatograph, mass spectrometer and multiple-ion detection controller have been described in an earlier paper [10].

### *Sample treatment and quantification*

On receipt of urine and plasma samples (from twelve individuals), eight untreated 500-$\mu$l aliquots were transferred to microcentrifuge tubes which were then placed in a light-proof box and frozen at $-18°$C. Two aliquots were analyzed immediately (without freezing), the others (in duplicate) at three, six and nine months after receipt. At the time of analysis, the aliquots were thawed, treated with deuterium-labelled internal standard mixtures, 500 $\mu$l of 10% sulfosalicylic acid (to precipitate protein and acidify the solution) and 100 $\mu$l of an anti-oxidant solution containing sodium bisulfite and EDTA. After centrifugation, the supernatant was processed and the metabolites quantified as described previously [10]. With each batch of samples, blank solutions (distilled water) and freshly prepared standard solutions (known quantities of authentic metabolites) were run in parallel with the biological samples.

*Calculations and statistics*

For each subject, the value obtained at 0 months (that is, the initial value before storage) was calculated and the percentage of the initial value remaining at three, six and nine months was determined. The results are summarized for the urinary and plasma acid metabolites in Figs. 1–4. Analysis of variance on the four groups in both plasma and urine was carried out (Table I).

## RESULTS

The results summarized in Figs. 1 and 2 show that there is a clear decline in the concentrations of most of the urinary metabolites over the nine-month period. Only PAA, MA and VMA are unchanged after nine months. pHPA concentration appears to increase with increasing storage time, possibly reflecting degradation of conjugates to free acid. MHPG and 5-HIAA have completely disappeared after nine months. Plasma metabolites, on the other hand, are essentially unchanged after nine months (Figs. 3 and 4), except for 5-HIAA which has completely decomposed (MHPG and DOPAC were not quantified in plasma). In Table I, the *F* values, significances and direction of change in the concentrations

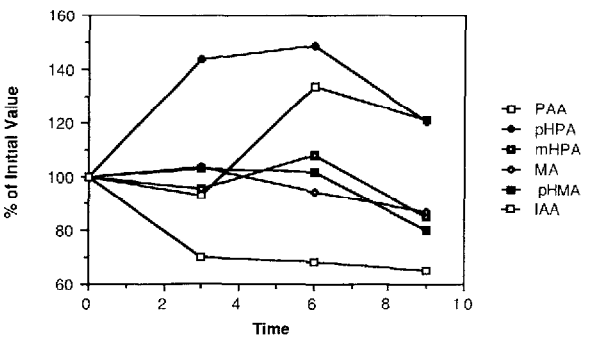


Fig. 1. Urinary trace amine metabolite concentrations remaining after storage for three, six and nine months (as percentage of initial value). Internal standard was added at time of analysis. For abbreviations see Table I.

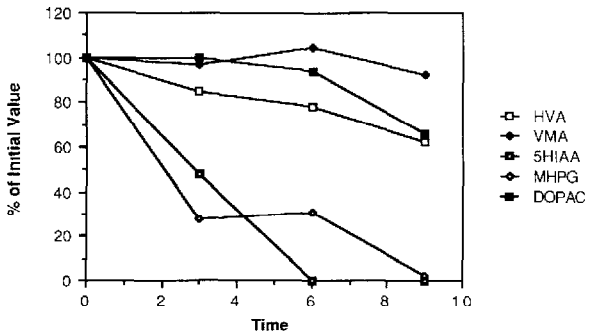


Fig. 2. Urinary catecholamine and serotonin metabolite concentrations remaining after storage for three, six and nine months (as percentage of initial value). Internal standard was added at time of analysis. For abbreviations see Table I

TABLE I

RESULTS OF ANALYSIS OF VARIANCE ON FOUR GROUPS

The groups are metabolite concentrations at 0, 3, 6 and 9 months on untreated samples (i.e., internal standards added only at time of analysis).

| Acid | Abbreviation | Urine | | | Plasma | | |
|---|---|---|---|---|---|---|---|
| | | Direction of change | $P^*$ | $F$ value** | Direction of change | $P^*$ | $F$ value** |
| Phenylacetic acid | PAA | ↑ | N.S. | 2.557 | ↑ | N.S. | 0.600 |
| *p*-Hydroxyphenylacetic acid | pHPA | ↑ | <0.005 | 5.204 | ↓ | N.S. | 1.651 |
| *m*-Hydroxyphenylacetic acid | mHPA | ↓ | <0.0001 | 17.874 | ↓ | <0.025 | 3.594 |
| Mandelic acid | MA | ↓ | N.S. | 1.888 | – | N.M. | N.M. |
| *p*-Hydroxymandelic acid | pHMA | ↓ | <0.005 | 8.556 | – | N.S. | 1.063 |
| Indoleacetic acid | IAA | ↓ | <0.005 | 5.088 | – | N.S. | 1.854 |
| Vanillylmandelic acid | VMA | – | N.S. | 1.229 | ↓ | N.S. | 0.581 |
| 5-Hydroxyindoleacetic acid | 5-HIAA | ↓ | <0.0001 | 854.846 | ↓ | <0.0001 | 469.441 |
| 3-Methoxy-4-hydroxyphenylethyleneglycol | MHPG | ↓ | <0.0001 | 76.684 | | N.M. | N.M. |
| 3,4-Dihydroxyphenylacetic acid | DOPAC | ↓ | <0.005 | 6.302 | | N.M. | N.M. |
| Homovanillic acid | HVA | ↓ | <0.0001 | 10.405 | ↓ | N.S. | 2.576 |

*N.S. = not significant; N.M. = not measured.

**$F$ values from one-way analysis of variance on four groups (0, 3, 6 and 9 months storage) of twelve samples each.

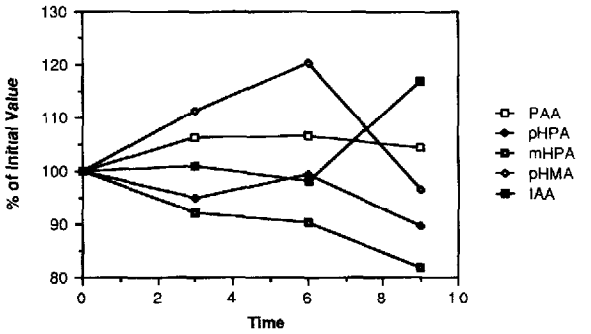


Fig. 3. Plasma trace amine metabolite concentrations remaining after storage for three, six and nine months (as percentage of initial value). Internal standard was added at time of analysis. For abbreviations see Table I.

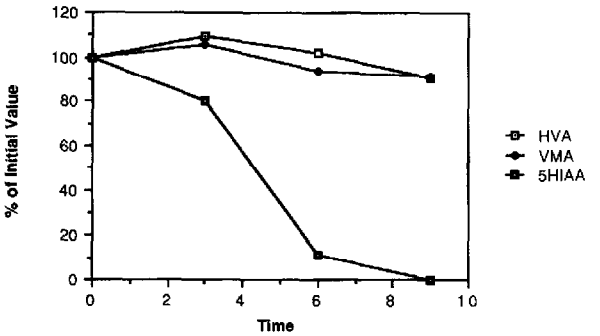


Fig. 4 Plasma catecholamine and serotonin metabolite concentrations remaining after three, six and nine months (as percentage of initial value). Internal standard was added at time of analysis. For abbreviations see Table I.

of the metabolites are summarized for analysis of variance on the four groups. In Table II, the initial values are compared with a weighted average of the means from several studies previously reported [11].

## DISCUSSION

The catecholamines, serotonin and their metabolites have been shown to be stable to varying degrees in stored biological material. Thus, urinary MHPG is stable when frozen at −20°C with a preservative at neutral pH for periods as long as 31 months [1–3]. In acidified urine, MHPG is less stable, although Moleman [4] has reported that MHPG is stable for at least a year if the anti-oxidants sodium metabisulfite and EDTA are present. However, under acidic conditions, conjugated MHPG is hydrolyzed almost completely to free MHPG when urine is stored at −14°C for two weeks [5]. MHPG in cerebrospinal fluid (CSF) at neutral pH also appears to be stable for up to 71 days when stored at −18°C, even without anti-oxidants [2].

5-HIAA appears to be less sensitive than MHPG to storage under acidic con-

TABLE II

INITIAL VALUES COMPARED WITH LITERATURE VALUES

| Metabolite* | Urine (mg per 24 h) | | Plasma (ng/ml) | |
|---|---|---|---|---|
| | This study | Literature | This study | Literature |
| PAA | 9.0 | 8.5 | 126.4 | 124.0 |
| pHPA | 20.8 | 18.6 | 54.4 | 69.0 |
| mHPA | 3.56 | 6.8 | 18.2 | 13.4 |
| MA | 0.30 | 0.23 | 3.3 | 13.6 |
| pHMA | 2.27 | 2.80 | 11.9 | 9.7 |
| HVA | 4.64 | 4.79 | 11.0 | 9.7 |
| VMA | 3.65 | 4.07 | 8.0 | 7.2 |
| IAA | 7.54 | 10.2 | 320.5 | 293.1 |
| DOPAC | 0.91 | 1.90 | Not measured | 3.5 |
| 5-HIAA | 4.38 | 4.53 | 31.0 | 11.4 |
| MHPG | 0.17 | 0.11 | Not measured | 3.55 |

*For abbreviations see Table I.

ditions, but has been widely reported to decompose on exposure to light. Fornstedt [6] has observed that standard solutions of 5-HIAA are stable at both pH 3 and 7 at −20°C when stored in a freezer in daily use only if the storage vials are covered in foil. Otherwise, 50% of the 5-HIAA decomposes in 100 days and 75% in 150 days. De Jong et al. [7] found standard solutions of 5-HIAA to be stable at 4°C and pH 0.7 for a week. The concentration of urinary 5-HIAA under the same conditions of storage decomposed to 70% of the initial value after 27 days. However, at pH 6.5 and −20°C, urinary 5-HIAA was found to be essentially unchanged after 27 days [7]. Verbiese-Genard et al. [8] tested the effects of dilute perchloric acid treatment on standard solutions of 5-HIAA and found no significant degradation during the first 24 h of storage, but after 72 h (at −20°C in the dark) 30% of the 5-HIAA had decomposed, although the use of anti-oxidants reduced decomposition to 15%. A similar sensitivity to light, particularly under acidic conditions, has been reported for 5-HIAA in CSF [9], although when stored at neutral pH and −70°C in the dark for 28 days no decomposition of 5-HIAA in CSF occurred.

VMA in urine [4] and HVA in CSF [9] or standard solutions [8] appear to be stable indefinitely even under acidic conditions.

The results of the present study have shown that only 5-HIAA and mHPA in plasma decompose significantly over nine months storage. In view of the earlier work cited above, the decomposition of 5-HIAA was to be expected, however, a statistically significant decline in the concentration of plasma mHPA is a surprise, although at 18% the loss is not great. It is worth noting that the PAA concentration does not increase significantly during long-term storage since about two thirds of total plasma PAA is present in conjugated form, probably as phenylacetylglutamine [12]. Evidently, the plasma environment is not conducive to appreciable hydrolysis of this conjugate.

The urinary environment, however, is conducive to the decomposition of most of the measured metabolites. 5-HIAA and MHPG disappear completely in nine months, which is not inconsistent with previous reports. VMA is stable, confirming an earlier study [4], as is MA, a new finding. The dopamine metabolites, DOPAC and HVA, show a marked decline in concentration, possible due to oxidation since no anti-oxidants had been added. As in plasma, urinary mHPA surprisingly declines significantly, although after nine months this decline amounts to only 15%. Urinary pHPA, on the other hand, increases significantly, which may reflect hydrolysis of sulfo-conjugated pHPA. That a corresponding increase was not observed for plasma pHPA may indicate that there is not an appreciable amount of sulfo-conjugated pHPA in plasma. A significant decline was observed for urinary IAA, but not for plasma IAA. It is not immediately obvious why this should be so. If the decomposition of so many urinary metabolites is due to oxidation during long-term storage one must conclude that urine, but not plasma, contains some as yet unidentified oxidizing materials. As in plasma, urinary PAA concentration does not significantly change, convincing proof of the stability of its conjugate, phenylacetylglutamine, which is present in urine in a ten- to hundred-fold excess over unconjugated PAA.

The lessons to be learned from this study are the following.

(i) For plasma samples, only 5-HIAA (and possibly MHPG) concentrations change significantly on long-term storage. As previous studies have shown, these compounds should be analyzed within a few weeks during which time they remain most stable at neutral pH, $-70°C$ and in the dark, preferably in the presence of anti-oxidants. The trace acids and other metabolites appear to be stable more or less indefinitely.

(ii) For urine samples, long-term storage of untreated samples results in massive decreases in the concentrations of 5-HIAA, MHPG and IAA. Much smaller but still significant declines in the concentrations of mHPA, pHMA, DOPAC and HVA are also observed. PAA, MA and VMA are stable at least for nine months.

These results are of particular importance when quantification is to be carried out by GC or high-performance liquid chromatography in which less than ideal internal standards must be used. The addition of anti-oxidants to urine samples may lessen or eliminate decomposition. However, analysis by GC–MS using deuterium-labelled analogues of the metabolites as internal standards is clearly to be preferred if samples must be stored for many months.

ACKNOWLEDGEMENTS

I wish to thank G. Wheatley and N. Pidskalny for expert technical assistance, Dr. D.A. Durden for supervision of the GC–MS analyses and Saskatchewan Health and the Medical Research Council of Canada for continuing financial support.

REFERENCES

1 G. Muscettola, W.Z. Potter and E.K. Gordon, Psychiatry Res., 4 (1981) 267.
2 R. O'Keeffe and B.W.L. Brooksbank, Clin. Chem., 19 (1973) 1031.

3 D. Pickar, D.R. Sweeney, J.W Mass and G.R. Heninger, Arch. Gen Psychiatry, 35 (1978) 1378.
4 P. Moleman, Clin. Chem., 31 (1985) 653.
5 F. Karoum, H. Lefèvre, L.B. Bigelow and E. Costa, Clin. Chim. Acta, 43 (1973) 127.
6 N. Fornstedt, Anal. Chem., 50 (1978) 1342.
7 J. de Jong, U.R. Tjaden, E. Visser and W.H. Meijer, J. Chromatogr., 419 (1987) 85.
8 N. Verbiese-Genard, M. Hanocq, C. Alvoet and L. Molle, Anal. Biochem., 134 (1983) 170.
9 M.A. Javors, C.L. Bowden and J.W. Maas, J. Chromatogr., 336 (1984) 259.
10 B.A. Davis, D.A. Durden and A.A. Boulton, J. Chromatogr., 374 (1986) 227.
11 B.A. Davis, J. Chromatogr., in preparation.
12 M.E. Martin, F. Karoum and R.J. Wyatt, Anal. Biochem. 99 (1979) 283.