Journal of Chromatography, 222 (1981) 161—169
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 719

LONGITUDINAL URINARY EXCRETION OF SOME "TRACE" ACIDS IN A HUMAN MALE

B.A. DAVIS and A.A. BOULTON*

Psychiatric Research Division, 508 University Hospital, Saskatoon, Saskatchewan S7N 0X0 (Canada)

(Received July 17th, 1980)

SUMMARY

Conjugated and unconjugated urinary levels of phenylacetic acid (PAA), *m*-hydroxyphenylacetic acid (*m*-HPA) and *p*-hydroxyphenylacetic acid have been determined for 24-h urine samples obtained from a single healthy male over a 28-day period. Gas chromatographic—electron-capture and mass spectrometric—integrated ion current techniques incorporating appropriate internal standards were used. The average urinary excretion values obtained were (in mg/24 h): PAA unconjugated 0.67, conjugated 96.6; *m*-HPA unconjugated 7.3, conjugated < 0.1; *p*-HPA unconjugated 22.4, conjugated < 1.2. Following the ingestion of appropriate deuterated amino acid precursors the expected urinary deuterated trace acids were identified and quantitated; in the case of deuterated phenylethylamine, *m*-HPA and *p*-HPA as well as PAA were identified and quantitated. This is the first evidence of phenylethylamine hydroxylation in the human. The longitudinal excretion of the trace acids was compared with that of the trace amines.

INTRODUCTION

Quantitative determinations of the levels of urinary phenylacetic acid (PAA), *m*-hydroxyphenylacetic acid (*m*-HPA) and *p*-hydroxyphenylacetic acid (*p*-HPA), the so-called "trace" acids, in the normal human have been reported [1—3]. These acids are the principal metabolites of the trace amines β-phenylethylamine (PE) and *m*- and *p*-tyramine (*m*-, *p*-TA) which have been claimed to be abnormally excreted in persons suffering from certain neurological and psychiatric disorders (see ref. 4 for a review).

Although the acid levels in the urines of large numbers of different control individuals have been determined [1—3], there has not been an investigation of the daily variations in these trace acids in the urine of a particular individual as has been the case for the trace amines [5]. In this paper, we describe the procedures and the results of the analysis of free and conjugated PAA, *m*-HPA

0378-4347/81/0000—0000/$02.50

and *p*-HPA in the urines of a human male (the same one as referred to in ref. 5) over a period of twenty-eight days (16 concurrent days in 1976 and 12 concurrent days in 1977). On certain days likely precursors of these acids (labelled with deuterium) were ingested and the urines analyzed for labelled acids.

Identification and quantitation of the acids was achieved by gas chromatography with electron-capture detection (GC—ECD), with periodic confirmation of the results by the gas chromatography—mass spectrometry—integrated ion current (GC—MS—IIC) technique.

MATERIALS AND METHODS

HPLC-grade solvents were purchased from Caledon Labs. (Georgetown, Canada), heptafluorobutyric anhydride (HFBA) and pentafluorobenzyl bromide (PFB-Br) from Pierce (Rockford, IL, U.S.A.). *m*- and *p*-hydroxyphenylacetic acids (Aldrich, Milwaukee, WI, U.S.A.) were deuterated by exchange reactions. Three exchanges involving heating 500 mg acid and 5 ml of 9% deuterium chloride in deuterium oxide at 110°C for 24 h yielded labelled acids containing no unlabelled acid, as determined by mass spectrometry. The major component in the *p*-HPA product contained four deuterium atoms (91%), two on the ring and two on the chain; in the *m*-isomer the major component contained five deuterium atoms (65%), three on the ring and two on the chain. Phenylacetic acid-α,α-D_2 was prepared by refluxing phenylacetonitrile in a 30% solution of NaOD in D_2O for 6 h. Incorporation was 98.3% D_2 and 1.7% D_1.

Twenty-eight 24-h urine samples starting with the first voiding each day were collected in May 1976 and 1977 in polyethylene bottles containing 10 ml conc. HCl. After recording the volume and pH, 250-ml aliquots of each urine were stored at —16°C until required for analysis. Precursors labelled with deuterium were ingested on day 2 (DL-phenylalanine-α,β-D_2, 3 g), day 6 (DL-tyrosine-D_2, 3 g), and days 13 and 24 (β-phenylethylamine-β,β-D_2), 200 mg each day.

At the time of analysis the samples were thawed and thoroughly mixed, 1-ml aliquots were removed and to each aliquot was added the appropriate internal standard homologue: *p*-hydroxyphenylpropionic acid (*p*-HPP) (27 μg) for GC—ECD analyses of *m*-HPA and *p*-HPA, and phenylpropionic acid (PPA) (70 μg) for analyses of PAA; similar quantities of deuterated *m*- and *p*-hydroxyphenylacetic acids were added for GC—MS—IIC analyses of *m*-HPA and *p*-HPA, and PAA-D_2 for analyses of PAA. The samples were saturated with NaCl (about 0.5 g) and extracted with ethyl acetate (3 × 2 ml); the extracts were then concentrated to 100—200 μl and transferred to 1-ml Reacti-vials (Pierce). Samples being analysed for PAA were treated at this stage with 50 μl triethylamine in order to form triethylamine salts of the acids and thus reduce losses due to evaporation. All samples were evaporated to dryness in a stream of nitrogen. Traces of water were removed by the addition of 100 μl benzene followed by evaporation to dryness in a stream of nitrogen.

For the quantitation of *m*-HPA and *p*-HPA, 150 μl of 15% HCl in methanol was then added to the samples. After 30 min at room temperature, the methanol was evaporated in a stream of nitrogen at 55°C, and the residue redried following the addition of benzene. To the residue was added 150 μl hexane

and 50 μl HFBA and this was then heated at 65°C for 1 h in a heating block. After cooling, 100 μl of phosphate buffer (pH 6.0) was added and the mixture vigorously shaken. The organic layer was withdrawn with a micropipette, the aqueous layer was then washed once with 100 μl hexane which after removal and combination with the original hexane layer was diluted to 500 μl with hexane.

For the quantitation of PAA, 200 μl of a benzene solution of PFB-Br (3.0%) and 18-crown-6 (3.0%) and a few milligrams of anhydrous potassium fluoride were added to the extract residue and heated at 50°C for 1 h [6]. The benzene solution was washed (2 × 200 μl) with water, evaporated in a stream of nitrogen almost to dryness and then dissolved in 0.5 ml hexane for GC—MS—IIC analysis or in 4.0 ml hexane for GC—ECD analysis.

For the GC quantitation, 0.5 μl (containing about 5 ng PAA or 50 ng *p*-HPA) was injected onto a GSC-SP2100 support-coated open-tube (SCOT) capillary column, 31 m in length, mounted in a Hewlett-Packard 5710A gas chromatograph fitted with an electron-capture detector (ECD). The flow-rate of carrier gas (5% methane in argon) through the column was 24 cm/sec. For the analysis of *m*-HPA and *p*-HPA as their HFB-methyl derivatives, the chromatograph conditions were: initial oven temperature 140°C isothermal for 2 min, then 4°C/min until the internal standard had been eluted (about 14 min), and finally 8°C/min to 260°C in order to purge the column of other uninteresting compounds. The injector temperature was 200°C and the detector temperature, 250°C. For the analysis of PAA as its PFB derivative, the chromatograph conditions were: initial oven temperature 195°C isothermal for 2 min, then 4°C/min to 260°C with baking at this temperature for 16 min. The injector temperature was 250°C and the detector temperature, 300°C.

For GC—MS quantitation, 1—5 μl of the hexane solution was transferred by means of a syringe to a solids injector, the solvent was allowed to evaporate, and the sample then injected onto a GSC-SP2250 SCOT column (57 m) mounted in a Hewlett-Packard 5710 gas chromatograph equipped with a flame ionization detector and coupled by means of a S.G.E. jet separator to an A.E.I. MS 902S mass spectrometer. The mass spectrometer was focused on the exact masses of the molecular ions of the derivatives of unlabelled acid and deuterated acid; as the sample was eluted from the column into the mass spectrometer source, the signal from each was recorded alternately as the instrument switched from one mass to the other. This procedure, the so-called integrated ion current (IIC) technique, has been described previously for the quantitation of trace amines [7]. The above described procedures are summarized in Fig. 1. Blanks were determined by carrying 1 ml of 2 *N* HCl through the entire procedure. Conjugated urinary acid levels were determined in an identical manner after hydrolysis of 1 ml urine for 60 min at 100°C following the addition of 0.5 ml conc. HCl. The conjugated acid values were calculated by subtracting the unconjugated value from that of the hydrolyzed urine.

Known amounts (10 ng to 100 μg) of authentic acids were also carried through the above procedures in order to calculate correction factors; these take into account differential extraction, derivatization, and detector response of the acids and internal standards. Both procedures yield linear calibration curves at least in the range 10^{-8}—10^{-4} g.

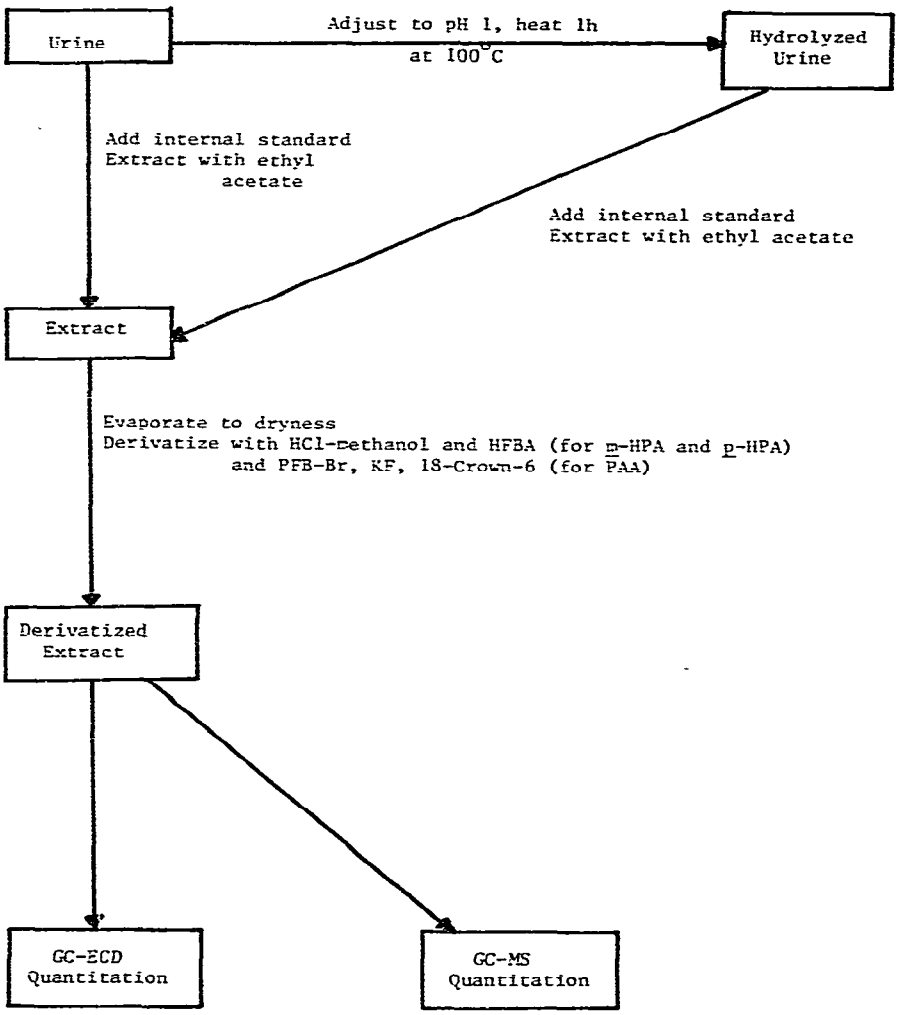

Fig. 1. Schematic outline for the isolation and quantitation of some trace acids.

The presence and amount of formed deuterated acids following the ingestion of the deuterated precursors was determined mass spectrometrically. First, the HC area ratio for the masses of the derivatives of the D_2-acid and non-labelled acid (designated D_2/H ratio) was determined on authentic non-deuterated acid. This provides the isotopic contribution of non-deuterated acid to the signal for the deuterated acid. The D_2/H ratio was then determined for derivatized samples isolated from the urine extracts. After subtraction of the isotopic contribution from the non-deuterated acid it is possible to calculate the absolute amount of deuterated acid present since the total amount of acid is known from the GC analysis and the ratio of deuterated to non-deuterated acid is known from the GC—MS—HC quantitation. In the case in which phenylalanine-α,β-D_2 was the precursor, the product acids contain only one deuterium atom, so the ratio measured was D_1/H.

RESULTS

The urinary excretion profiles as determined by GC—ECD for conjugated PAA and unconjugated *m*-HPA and *p*-HPA along with earlier conjugated amine values are shown in Figs. 2, 3 and 4. [The unconjugated amine values (see

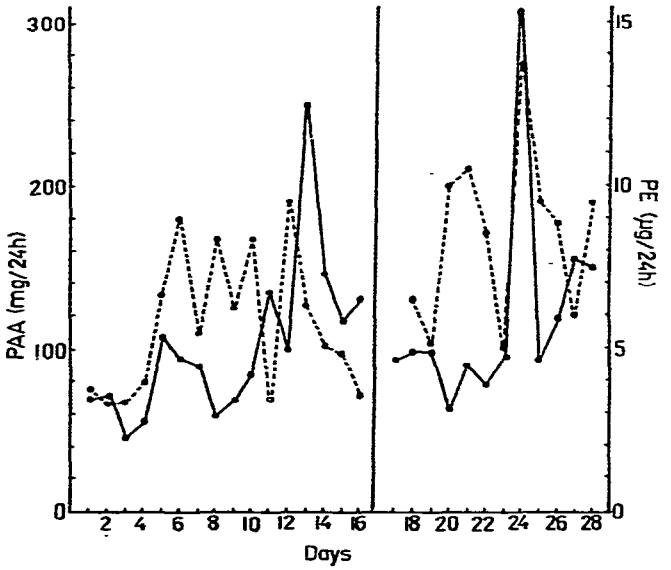

Fig. 2. Urinary excretion of conjugated phenylacetic acid (●——●) and conjugated phenylethylamine (○ — — — ○) in a human male.

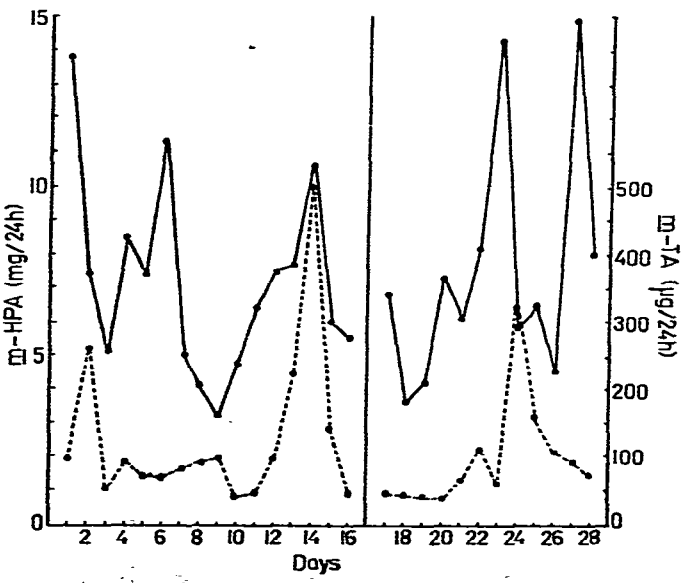

Fig. 3. Urinary excretion of unconjugated *m*-hydroxyphenylacetic acid (●——●) and conjugated *m*-tyramine (○ — — — ○) in a human male.

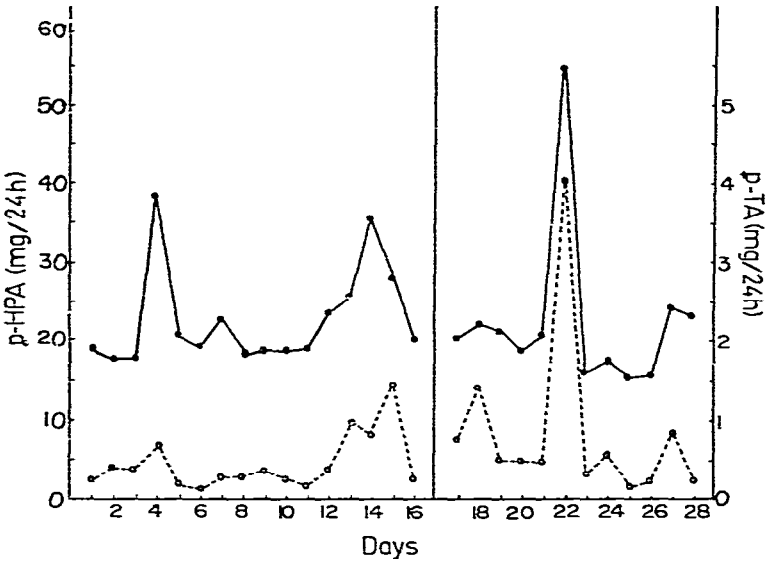

Fig. 4. Urinary excretion of unconjugated *p*-hydroxyphenylacetic acid (•——•) and conjugated *p*-tyramine (○ — — — ○) in a human male.

Figs. 2—4 in ref. 5) were not plotted because they show relatively little variation from day to day.] Unconjugated PAA in this particular individual constituted less than 1% of total PAA and so has not been included in the figure. The average unconjugated PAA level was 0.67 mg/24 h compared to 96.6 mg/24 h conjugated PAA. This subject normally excreted insignificant amounts of conjugated *m*-HPA or conjugated *p*-HPA except on day 1 in this series when 19% of the total *m*-HPA and 20% of the total *p*-HPA was conjugated. The mean values ± standard error of the means, and the ranges of these urinary trace acids are summarized in Table I.

TABLE I

URINARY EXCRETION OF PHENYLACETIC ACID AND *m*- AND *p*-HYDROXYPHENYLACETIC ACIDS IN A HUMAN MALE

Values using the GC—ECD technique represent daily excretion over the 28-day period (mean ± S.E.M.) of the indicated acid except in the first line where days 13 and 24 (i.e., the days of ingestion of phenylethylamine) are omitted.

Acid	Amount (mg/24 h)	Range (mg/24 h)
Phenylacetic (conjugated)	96.4 ± 5.6	45.5—155.6
Phenylacetic (conjugated)	110.0 ± 10.6	45.5—306.8
m-Hydroxyphenylacetic (unconjugated)	7.3 ± 0.6	3.2— 14.9
p-Hydroxyphenylacetic (unconjugated)	22.4 ± 1.5	15.3— 54.0

The presence and amounts of deuterated acids formed from the ingested deuterated precursors are listed in Table II.

When standard solutions of the acids were carried through the procedures,

TABLE II

URINARY DEUTERATED TRACE ACIDS ARISING FROM LABELLED PRECURSORS

— = not present; nd = not determined. Values obtained using GC—MS—IIC procedure. Figures in parentheses represent percentage of labelled acid as proportion of total acid excreted.

Precursors	PAA (mg) (conjugated)	*m*-HPA (mg) (unconjugated)	*p*-HPA (mg) (unconjugated)
Phenylalanine-D_2 (day 2)	2.5 (3.5)	—	—
p-Tyrosine-D_2 (day 6)	—	—	2.7 (14.5), day 6 4.6 (20.6), day 7 0.9 ((5.0), day 8
Phenylethylamine-D_2 (day 13)	31.9 (12.8)	0.031 (0.4)	0.026 (0.1)
Phenylethylamine-D_2 (day 24)	36.5 (11.9)	nd	nd

the results obtained indicated that correction factors needed to be applied in some cases. With respect to the GC—ECD analyses of PAA for example, the correction factor was 0.658; this is presumably in part at least because PPA is not a perfect internal standard and as such does not extract, derivatize or activate the electron-capture detector in exactly the same way as PAA. In the GC—MS analyses, the correction factor was higher at 0.825. We presume that the reason for this being less than 1.0 relates to the fact that during hydrolysis in strong acid some back-exchange of the deuterated internal standard occurs.

DISCUSSION

From Figs. 2, 3 and 4, and Table I, it can be seen that in this particular individual there is a considerable variation in the daily excretion of conjugated PAA and unconjugated *m*-HPA and *p*-HPA. The excretion of unconjugated *p*-HPA appears to correlate quite well with conjugated *p*-TA (see also ref. 5) but much less so between unconjugated *m*-HPA and *m*-TA. There appears to be little, if any, correlation between the excretion of conjugated PE and conjugated PAA.

The ingestion of deuterated phenylalanine and *p*-tyrosine does not appear to have exerted any particularly significant effect on the total urinary excretion of *m*-HPA, *p*-HPA or PAA; the ingestion of PE-D_2, on the other hand, influenced markedly the output of PAA on days 13 and 24. In this latter case, however, it is interesting to note that although the conjugated urinary PAA output increased from an average of about 100 mg/day before the ingestion of 200 mg phenylethylamine-D_2 to 250 mg/day on day 13 and 306 mg/day on day 24 (when the PE-D_2 was consumed), only 12% of that urinary PAA was deuterated. This means that only 30—40 mg of the 250 or 306 mg was labelled; consequently more than 200 mg of extra unlabelled conjugated PAA was ex-

creted on days 13 and 24. This is more than double the daily average and seems to point to the induction of much greater than normal amounts of urinary PAA being formed when PE had been ingested.

A complicating factor in determining the actual amount of urinary deuterated conjugated acids present in the urine was the back-exchange between deuterium and hydrogen atoms which occurred when the acidified urine was heated at 100°C for 60 min. In standard solution, 17.5% of the D_2-acid was lost under these conditions [90% of this became D_1-acid and 10% unlabelled (i.e. hydrogen) acid]. For shorter hydrolysis times, this loss is reduced but in these cases the hydrolysis is incomplete.

Although the ingestion of deuterated amino acid precursors did not significantly increase the total urinary excretion of the trace acids, these acids labelled with deuterium were identified and quantitated in the urine in many cases (see Table II). After eating 3 g of phenylalanine-D_2 on day 2 for example, the excreted conjugated PAA was 3.5% labelled, although no labelled *m*-HPA or *p*-HPA was identified. Ingestion of labelled *p*-tyrosine on day 6 produced labelled unconjugated *p*-HPA on days 6, 7 and 8 amounting to 14.5%, 20.6% and 5.0% respectively of the total unconjugated *p*-HPA excretion (see Table II). Ingestion of PE-D_2, as stated earlier, produced substantial quantities of labelled conjugated PAA, but in addition small but easily measurable amounts of labelled *m*-HPA and *p*-HPA. From this it is permissible to conclude that ring hydroxylation of PE occurs in man yielding *m*- and *p*-TA which are then oxidatively deaminated to *m*-HPA and *p*-HPA as is the case in the rat [8]. It is interesting to note, as has been noted in an earlier publication [2], that there can be quite a considerable lag (up to 3 days in some cases) between the ingestion of a precursor and its subsequent appearance, as a metabolite, in the urine.

A comparison of the values obtained in this longitudinal study on a single individual with those from earlier studies in which many different individuals were assessed gives good agreement. Thus Martin et al. [1] obtained a value of 137.4 ± 15.8 mg/24 h for total urinary PAA using a mass fragmentographic method and Sandler et al. [3] reported 119.8 ± 15.0 mg/24 h using a gas chromatographic procedure. The average value obtained in this study was 97.4 mg/24 h, as determined by GC—ECD and confirmed by GC—MS—IIC (see Table III for a comparison of the two procedures). In the case of *m*-HPA the value reported here (7.3 mg/24 h) agrees quite well with that listed by Sandler et al. [3] (5.8 mg/24 h) and for *p*-HPA, our value (22.4 mg/24 h) similarly agrees closely with that listed by Sandler et al. [3] (21.3 mg/24 h) although it is lower than that listed by Boulton [2] in an earlier study using a fluorimetric procedure.

Finally there is the question of the name "trace" in reference to these particular amines and their acid metabolites. Whilst it is a fact that in mammalian tissues PE, *m*-TA and *p*-TA are found in very small amounts [9] this is not so in some invertebrates [10]. It is also not so in mammalian tissues in the presence of certain drugs such as monoamine oxidase inhibitors [11]. In the case of the acids PAA, *m*-HPA and *p*-HPA it is again a fact that in mammalian brain they are found in small amounts [12—14], in urine however they are found in substantial amounts approximately equivalent to the acid

TABLE III

COMPARISON OF RESULTS OBTAINED BY GC—ECD AND GC—MS—IIC IN SELECTED URINE SAMPLES

See text for details.

Acid (mg/24 h)	Day	GC—ECD	GC—MS—IIC
PAA	1	70.7	58.3
	2	74.7	85.2
	16	132.5	119.9
	21	89.7	73.4
	26	117.1	116.4
m-HPA	2	7.4	8.1
	3	5.1	4.0
	22	8.2	7.4
p-HPA	2	17.5	15.2
	3	17.5	13.3
	22	54.0	50.6

metabolites of the putative neurotransmitters (homovanillic acid, dihydroxyphenylacetic acid, 5-hydroxyindole acetic acid, etc.) and this indicates that in the body the so-called trace amines are in a state of rapid and dynamic turnover [4, 9, 15]. This suggests that they play an important role in metabolism and may perhaps be involved in the modulation or propagation of nervous impulses as has been proposed [4, 9].

ACKNOWLEDGEMENTS

We thank Dr. D.A. Durden for setting up the GC—MS system and Ms. E.E. Johnson for the GC—MS analyses, and Saskatchewan Health and the Canadian Medical Research Council for continuing financial support.

REFERENCES

1 M.E. Martin, F. Karoum and R.J. Wyatt, Anal. Biochem., 99 (1979) 283.
2 A.A. Boulton, in Progr. Neurogenetics, 1 (1967) 437.
3 M. Sandler, C.R.J. Ruthven, B.L. Goodwin, G.P. Reynolds, V.A.R. Rao and A. Cooper, Nature (London), 278 (1979) 357.
4 A.A. Boulton, in L. Battistin, G.A. Hashim and A. Lajtha (Editors), Neurochemistry and Clinical Neurology, Vol. 39, Liss, New York, 1980, pp. 291—303.
5 N.D. Huebert and A.A. Boulton, J. Chromatogr., 162 (1979) 169.
6 B.A. Davis, Anal. Chem., 49 (1977) 832.
7 D.A. Durden, S.R. Philips and A.A. Boulton, Can. J. Biochem., 51 (1973) 995.
8 A.A. Boulton, L.E. Dyck and D.A. Durden, Life Sci., 15 (1974) 1673.
9 A.A. Boulton, Int. Rev. Biochem., 26 (1979) 179.
10 H.A. Robertson and A.V. Juorio, Int. Rev. Neurobiol., 19 (1976) 173.
11 S.R. Philips and A.A. Boulton, J. Neurochem., 33 (1979) 159.
12 F. Karoum, J.C. Gillin and R.J. Wyatt, J. Neurochem., 25 (1975) 653.
13 D.A. Durden and A.A. Boulton, J. Neurochem., in press.
14 D.A. Durden, private communication.
15 D.A. Durden and S.R. Philips, J. Neurochem., 34 (1980) 1725.