A SPECIFIC RADIOENZYMATIC ASSAY FOR DIHYDROXYPHENYLALANINE (DOPA). PLASMA DOPA MAY BE THE PRECURSOR OF URINE FREE DOPAMINE

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- 1 A sensitive radioenzymatic assay was developed, in which DOPA is enzymatically decarboxylated to dopamine and the latter converted to [^3H]-methoxytryamine in the presence of [^3H]-S-adenosyl-L-methionine and catechol-*o*-methyltransferase.
- 2 The assay was specific for DOPA, and was sensitive to 50 pg/ml.
- 3 Endogenous DOPA was found to be present in the plasma of eight human volunteers at a concentration of 10.46 ± 2.42 nmol/l.
- 4 Simultaneous urine collections in the same subjects showed a free dopamine excretion of 68.88 ± 17.70 nmol/h. There was a significant correlation ($P < 0.01$) between plasma DOPA concentration and urine free dopamine excretion ($r = 0.84$).
- 5 After the oral administration of 250 mg levodopa, plasma DOPA and urine dopamine both increased by a similar proportion (98 ± 8.4 -fold, and 93.4 ± 6.9 -fold respectively). These compare with an increase in plasma dopamine of only 26 ± 15 -fold ($P < 0.01$).
- 6 Following the oral dose of DOPA, the increase in plasma DOPA, but not plasma dopamine, could account for the increase in urine dopamine. The calculated clearance of plasma DOPA by renal decarboxylation to dopamine was 114 ± 20 ml/min.
- 7 This is not significantly different from the apparent clearance of endogenous DOPA by renal decarboxylation to dopamine, and suggests that there is adequate renal decarboxylase activity for DOPA to be the precursor for renal dopamine formation.

Introduction

In man, dopamine is the major catecholamine in urine (Sourkes & Drujan, 1957). Its origin is uncertain because there is insufficient dopamine in plasma to provide the amount excreted, suggesting that intrarenal dopamine formation is the most likely source (Alexander, Gill, Yamabe, Lovenberg & Keiser, 1974). Since dopamine in the sympathetic nervous system and the brain is derived from DOPA, and the enzyme aromatic amino acid decarboxylase is present in renal tubules (Goldstein, Fuxe & Hokfelt, 1972), the most probable precursor for renal dopamine formation is circulating DOPA. Existing assays are not sufficiently sensitive to determine normal circulating levels of DOPA in human plasma. A more sensitive assay has been developed with the objective of studying the source of urinary dopamine. The catechol-*o*-methyltransferase preparation used for the radioenzymatic assay of catecholamines also contains appreciable amounts of aromatic amino acid decarboxylase activity (Ben Jonathan & Porter, 1976). Accurate determination of dopamine levels in the sample where DOPA is also present requires the inclusion in the assay incubation mixture of an aromatic amino acid decarboxylase inhibitor (Peuler &

Johnson, 1977). The presence of the decarboxylase has been exploited to develop a sensitive radioenzymatic assay for the determination of DOPA in plasma and urine.

The assay has been used to measure the relationship between plasma DOPA concentration and urine dopamine excretion, in order to estimate whether there is sufficient DOPA in plasma to be the major precursor for urine dopamine. The measurements were made both before and after the administration of an oral DOPA load.

Method

Materials and analytical method

Catechol-*o*-methyltransferase was prepared from rat livers according to the method of Axelrod & Tomchick (1958); this preparation also contained aromatic amino acid decarboxylase activity. [^3H]-S-adenosyl-L-methionine (33 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. Solvents were obtained from BDH, tetraphenylboron

sodium from Aldrich and all other chemicals were purchased from Sigma.

Unextracted plasma samples, and urine diluted 1:50 in water, were used for assay. For the assay of the DOPA and dopamine content of a sample, eight 100 μ l aliquots were required. To 4 of these was added 10 μ l 0.01 M HCl; to the remainder was added 10 μ l 0.01 M HCl containing 1 ng of either DOPA or dopamine as internal standard. Blanks consisted of 100 μ l distilled water. The aliquots for DOPA estimation were incubated at 37°C for 1 h with 50 μ l of a mixture containing 25 μ l enzyme preparation, 400 mM Tris, 120 mM magnesium chloride (MgCl_2), 40 mM ethyleneglycol tetraacetic acid (EGTA), 5 mM reduced glutathione (GSH), 10 μ M pyridoxal phosphate and 5 μ Ci [^3H]-S-adenosyl-L-methionine (SAME). A solution of Tris, MgCl_2 and EGTA in these proportions has the desired pH of 8.4; the incubation mix for each assay was prepared by dissolving the GSH and pyridoxal phosphate in this solution and adding the enzyme and [^3H]-SAME. The aliquots for dopamine estimation were incubated with an identical mixture containing also 0.1 mM benzylhydroxylamine. After the incubation, the tubes were placed on ice and 50 μ l of the following mixture was added: 1 M sodium borate, pH 8.0, 1.5 mM sodium tetraphenylboron and 60 μ M methoxytyramine. Diethyl ether (2 ml) was dispensed into the tubes, which were then vortexed for 15 s and briefly centrifuged. The aqueous layer was frozen by placing the tubes in a freezing mixture of acetone and solid carbon dioxide, and the ether layer of each tube was decanted into a fresh set of tubes containing 25 μ l 0.1 M HCl and 5 mM methoxytyramine. The aqueous layer was discarded. The tubes were vortexed for 15 s and centrifuged briefly. The aqueous layer was again frozen, and on this occasion the ether layer in each tube was discarded. After thawing, the aqueous layer was spotted on Whatman t.l.c. plates containing a pre-absorbent origin and a fluorescent indicator. The plates were developed in chloroform, methanol and 70% ethylamine 32:6:4. The methoxytyramine band was marked under u.v. light and scraped into tubes containing 0.5 ml 0.05 M ammonium hydroxide. These tubes were shaken for 10 min and then 0.4 ml of 2 M Tris, pH 9.6, was added to each tube. Acetic anhydride (25 μ l) was added to each tube, which was then vortexed for 20 s. The tubes were allowed to stand for 30 min, when 100 μ l of glacial acetic acid was added. All tubes then received 10 ml of toluene and were vortexed for 15 s. After centrifugation the aqueous layer was frozen and the toluene decanted into scintillation vials containing 0.4 ml Permafluor (Packard). The DOPA concentration (ng/ml) of the original sample was calculated as follows:

$$\frac{\text{CPM}_1 - \text{CPM}_2}{\text{CPM}_3 - \text{CPM}_1} \times 10 \text{ for plasma samples}$$

$$\frac{\text{CPM}_1 - \text{CPM}_2}{\text{CPM}_3 - \text{CPM}_1} \times 500 \text{ for urine samples}$$

where CPM_1 = the mean counts per minute for the duplicate aliquots assayed without internal standard with the first mixture (no decarboxylase inhibitor)

CPM_2 = mean counts per minute for the aliquots assayed with the second mixture (including the decarboxylase inhibitor)

CPM_3 = counts per minute for the aliquot including the DOPA internal standard

Studies in normal volunteers

Measurements of plasma and urine DOPA and dopamine were made in eight healthy volunteers (six male and two female). Subjects gave informed consent, and the studies were approved by the Ethics Committee of the Royal Postgraduate Medical School. Before the start of the studies, the subjects fasted for 10 h. They remained supine in a quiet clinical laboratory except when they were required to provide specimens of urine.

Blood samples were drawn from a forearm venous cannula (19 gauge butterfly, Abbott), whose patency was maintained by intermittent injection of 0.9% saline containing 1U/ml of heparin. Blood (5 ml) was placed in a chilled lithium heparin tube, and the plasma separated within 30 min at 4°C. This was stored at -20°C, and assayed within 2 weeks. Urine was passed directly into plastic containers, and the samples were stored at 4°C after acidification with 1M HCl to pH 2 to 3.

The endogenous plasma DOPA and dopamine levels were estimated on two samples drawn 1 h apart. A 1 h urine collection was made during this same period in order to estimate the DOPA and dopamine excretion. At the end of this hour, the subjects received an oral dose of 250 mg of levodopa. Further blood samples were drawn at the midpoint of each of the following 4 h. Hourly urine collections were made for 4 h after the administration of levodopa (in addition to the hour's collection before dosing).

Subjects were taking a free sodium diet, and their sodium excretion was not measured.

Results

The assay was linear from 0 to 50 ng of DOPA (Figure 1). This was estimated by adding to the blanks 10 μ l 0.01M HCl containing increasing amounts of DOPA. The sensitivity (defined as the smallest concentration of DOPA which could be distinguished with 95% confidence from the background) was 50 pg/ml (0.25 nmol/l). For plasma, the sensitivity was sometimes lower, since the recovery of standards was variable: 1 ng may yield from 9000 to 21000 counts/min. The

Figure 1 Standard curve for DOPA. Increasing amounts of DOPA were assayed as described in the text. Values shown are the mean $\pm$ s.d. of 6 estimations.

recovery from urine was rarely less than from distilled water: 1 ng yielded 23000 to 25000 counts/min.

The precision of the assay was estimated using plasma from the subject with the lowest resting plasma DOPA concentration: the intra- and inter-assay coefficients of variation were 3.9% and 6.2% respectively. The specificity was checked by adding to the blanks 10 μ l 0.01M HCl containing 50 ng of possible interfering compounds: noradrenaline, adrenaline, dopamine, tyramine, epinine, octopamine, tyrosine, homovanillic acid, α methyl dopa and dihydroxyphenylacetic acid. Each was assayed in duplicate, in the presence and absence of the decarboxylase inhibitor. None gave higher counts than the blank, except adrenaline and α methyl dopa, which gave 763 ± 31 counts/min and $21,463 \pm 578$ counts/min respectively. These are less than 0.1% and 5% of the counts from 50 ng of DOPA, and would not interfere with the measurement of DOPA in biological samples where adrenaline is present in smaller concentrations than DOPA, and α methyl dopa is not present at all. The decarboxylase inhibitor, benzylhydroxylamine, at the concentration employed in the assay completely prevented the conversion of DOPA to dopamine: in its presence, 50 ng of DOPA gave no more counts than the blank.

For dopamine, the sensitivity was 0.2 nmol/l, and the assay was linear within the same range as for DOPA. Recovery from plasma was again less than from distilled water (or diluted urine samples) and dopamine was not detectable in some plasma samples from which recovery was low. Intra- and inter-assay coefficients of variation were 4.1% and 6.4%.

Results in volunteers

Table 1 shows the basal DOPA and dopamine levels in plasma and urine samples obtained from healthy

Table 1 Resting levels of DOPA and dopamine in plasma and urine before oral administration of L-DOPA

Subject	DOPA		Dopamine	
	Plasma (nmol/l)	Urine (nmol/h)	Plasma (nmol/l)	Urine (nmol/h)
1	7.11	18.61	—	36.63
2	8.12	18.84	0.38	50.96
3	8.63	17.46	0.61	73.25
4	10.15	21.35	0.45	70.07
5	10.66	26.97	—	76.44
6	12.18	23.32	0.53	66.88
7	13.19	24.66	0.49	82.81
8	13.71	28.04	0.31	92.36
Mean	10.46	22.38	0.35	68.68
s.d.	2.42	3.97	0.23	17.7

volunteers. Plasma DOPA concentration before the administration of levodopa was 10.46 ± 2.42 nmol/l. As shown in Figure 2, there was a highly significant correlation between the resting plasma DOPA concentration and urine dopamine excretion in the group. There was no significant correlation between the resting plasma dopamine concentration and urine dopamine ($r = -0.16$).

Following an oral DOPA load the plasma and urine measurements were repeated at hourly intervals. The dose of 250 mg was sufficient to increase the DOPA and dopamine levels in plasma and urine to several times their resting values (Figure 3). The maximal increase in plasma DOPA and urine dopamine were by a similar proportion (98 ± 8.4 -fold and 93.4 ± 6.9 -fold, respectively) ($P > 0.1$), but plasma dopamine increased by only 26 ± 15 -fold ($P < 0.01$). Thus the increased urine dopamine excretion is likely to have been derived from the increase in plasma DOPA rather than plasma dopamine concentration.

Figure 2 Relation of plasma DOPA to urine dopamine in eight healthy volunteers. Each symbol represents one subject. $y = 4.1 + 6.17x$, $r = 0.85$, $P < 0.01$.

Figure 3 Change in DOPA and dopamine levels in plasma (lower trace) and urine (upper trace), following the oral administration of 250 mg levodopa to the eight subjects. Each point represents the mean $\pm$ s.d. for the group.

Both before and after the oral DOPA, the calculated renal clearance of plasma dopamine required to account for urine dopamine would be greater than the total renal plasma flow estimated from the body weight and height of each subject (Smith, 1951). From the data in table 1, it is possible to calculate the renal clearance of plasma DOPA (by decarboxylation to dopamine) required to supply the amount of dopamine excreted in the urine. The resting figure is 109 ± 17 ml/min. Following the DOPA load, the urine DOPA and dopamine lagged behind the increase in the plasma concentrations, for the first 2 h (Figure 3). Thereafter they regained the same relationship as before with a calculated clearance of plasma DOPA into urine dopamine of 114 ± 20 ml/min. This is not significantly different from the resting figure, and both are much lower than the estimated renal plasma flow (637 ± 49 ml/min).

Discussion

The assay described has similar sensitivity for DOPA as the radioenzymatic assays for catecholamines, on which much of the present assay is based (Peuler &

Johnson, 1977; Da Prada & Zurcher, 1976). This has made it possible to demonstrate that DOPA is a normal constituent of human plasma, and is present at a higher concentration than the catecholamines. It seems unlikely that plasma DOPA is of importance as a precursor of catecholamines in the brain and sympathetic nervous system; although DOPA would enter the synthetic pathway distal to the rate limiting step of tyrosine hydroxylase, the concentration of tyrosine in plasma is many times higher than that of DOPA (Stein & Moore, 1954). So most DOPA in sympathetic nervous tissue is likely to be formed *in situ* from tyrosine, rather than being derived from plasma DOPA.

The present study has suggested that one possible role of plasma DOPA is as a precursor for dopamine formation in the kidney. If this occurs in the renal tubules, where there is a high level of aromatic amino acid decarboxylase activity, the DOPA could not be derived from circulating tyrosine since tyrosine hydroxylase is not present in these cells (Nagatsu, 1973). As has been previously reported, the dopamine in plasma can account for only a small proportion of the dopamine excreted in urine (Oates, Ball, Perkins & Lee, 1979). When, however, the apparent clearance of plasma DOPA by renal decarboxylation was calculated on the assumption that urine free dopamine is mainly derived from plasma DOPA, this was found to be a little over 100 ml/min. This represents less than one quarter of the probable renal plasma flow in these subjects, and suggests that renal decarboxylation of less than one quarter of DOPA in renal plasma is sufficient to supply the free dopamine excreted in urine.

That decarboxylation of DOPA does occur to this extent is suggested by the calculations made following the oral DOPA load. Since both plasma DOPA and urine dopamine then increased by a similar proportion, while plasma dopamine did not, it can be concluded that the increased dopamine excretion was derived from the increase level of plasma DOPA. (Once again, calculations based on the estimated renal plasma flow show that plasma dopamine could not supply more than a small fraction of urine dopamine. Even total renal clearance of the resting plasma dopamine would account for less than 10% of the resting urine dopamine; this figure would be even smaller after the oral DOPA, which increased the urine dopamine by a higher proportion than the plasma dopamine.) The apparent renal clearance of DOPA by decarboxylation to dopamine did not change significantly following DOPA administration; if, then, the above argument is correct that the increased urine dopamine excretion following oral DOPA is derived from plasma DOPA, it follows that the kidney has sufficient decarboxylase activity for the endogenous plasma DOPA to supply the endogenous urine dopamine.

This demonstration does not prove the origin of urine dopamine. This may require tracer experiments using radioactively labelled DOPA (or other possible precursors), and studies of arterio-venous differences across the kidney. If DOPA is the precursor of urine dopamine, a reduction in urine dopamine excretion would be expected following administration of an inhibitor or aromatic amino acid decarboxylase. This has been reported in subjects receiving the drug, carbidopa (Ball & Lee, 1977). Although only a small fall in urine dopamine excretion was noted in that study, this may have been a dose effect or due to poor concentration of the drug in renal tubular cells: it would be desirable to study the effects of the drug at several doses, and show that the highest dose was effective at inhibiting renal decarboxylation of exogenously administered DOPA.

The significant correlation between endogenous plasma DOPA concentration and urine dopamine excretion (Figure 2) suggests that the plasma DOPA concentration may be an important factor in deter-

mining urine dopamine excretion. Sodium intake is also known to influence dopamine excretion (Alexander *et al.*, 1974; Faucheux, Buu & Kuchel, 1977; Oates *et al.*, 1979); it will be of interest to investigate whether these two factors operate independently, or whether changes in sodium intake may modulate dopamine excretion via changes in plasma DOPA concentration.

The availability of a specific and sensitive assay for plasma DOPA may have another, quite different use—namely in the study of the pharmacokinetics of levodopa administered to Parkinsonian patients. However, it would be advisable to dilute the samples, since large amounts of DOPA can lead to inactivation of the aromatic amino acid decarboxylase enzyme (O'Leary & Baughn, 1975). Although high concentrations of the coenzyme, pyridoxal phosphate, protect against this, it would be necessary to check that the complex kinetics of the double enzyme reaction does not lead to loss of linearity when assaying high concentrations of substrate.

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