Biochemical Diagnosis of Pheochromocytoma by Simultaneous Measurement of Urinary Excretion of Epinephrine and Norepinephrine

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The utility of specific assay of urinary catechols in pheochromocytoma diagnosis was examined by reviewing our data on the investigation of pheochromocytoma in a population of 2476 patients investigated over a six-year period. We used specific gas chromatographic/mass-spectrometric (GC/MS) analysis for the simultaneous measurement of norepinephrine, dopamine, and the neuronal metabolites 3,4-dihydroxyphenylglycol (DHPG) and 3,4-dihydroxyphenylacetic acid (DOPAC) in all samples; the last two years of data collection (from 1101 patients) also included the specific GC/MS assay of epinephrine. The importance of assaying epinephrine as well as norepinephrine was shown by these latter data. During this latter period, 19 of 1101 patients were found to have pheochromocytoma; of these, nine had tumors that exclusively secreted norepinephrine, six had tumors that exclusively secreted epinephrine, and four exhibited excess production of both norepinephrine and epinephrine. Neither dopamine nor DOPAC was useful in the diagnosis of pheochromocytoma. A substantial proportion of patients may have uniquely epinephrine-secreting pheochromocytomas, previously considered a rarity. Thus we recommend that the biochemical testing for pheochromocytoma include the specific measurement of both norepinephrine and epinephrine.

Additional Keyphrases: gas chromatography/mass spectrometry, dopamine, catecholamines

Recent reports have drawn attention to the protean nature of the presentation of pheochromocytoma and stressed the need for more definitive biochemical diagnosis (1). This need has been highlighted further by the proposal that all patients found incidentally to have an adrenal mass on abdominal computed tomography scan should be screened for pheochromocytoma (2). The lack of unanimity regarding screening procedures and the high prevalence of pheochromocytoma found at autopsy indicate a need for a reevaluation of usual diagnostic criteria (3, 4).

In 1988, using a large database of hypertensive patients with and without pheochromocytoma, we reported that 24-h urine sampling was superior to plasma sampling in the diagnosis of pheochromocytoma (5). In that study we found that some of the tumors produced large quantities of 3,4-dihydroxyphenylglycol (DHPG), normally the primary neuronal metabolite of norepinephrine (NE) (5). This observation led us to suggest a cautious approach to interpreting the NE/DHPG ratio, which has been proposed as an index of NE production by tumor vs neuronal origin (6); however, a diagnostically useful increase in specificity was gained by the simultaneous measurement of DHPG and NE (5).

In our original investigation, although we recommended the measurement of epinephrine as well as NE and DHPG (5), we had not developed a specific gas chromatographic/mass-spectrometric (GC/MS) assay for epinephrine and thus were unable to investigate the prevalence of pheochromocytomas that might secrete epinephrine uniquely (i.e., no increased production of NE). Earlier work had indicated that pure epinephrine-secreting tumors, although rare, do occur (4, 7–9) and may be a major feature of pheochromocytomas associated with multiple endocrine neoplasia type 2a (MEN 2a) (9). In one investigation, 5 of 14 diagnosed pheochromocytomas (36%) were uniquely epinephrine-secreting and did not cause hypertension (10). Another report of a uniquely epinephrine-secreting tumor suggests that such tumors can be associated with prevailing postural hypotension but that adrenal cortical stimulation with corticotropin may result in hypertensive crises (11).

The present study was designed to examine, in a large population of subjects suspected of having a pheochromocytoma, the diagnostic utility of daily urinary excretion rates for each of the major catecholamines—epinephrine, NE, and dopamine—and the principal neuronal metabolites DHPG and 3,4-dihydroxyphenylacetic acid (DOPAC). We routinely assess dopamine production as part of our tertiary referral service; because of occasional reports that dopamine measurement has a role in pheochromocytoma diagnosis (7, 12, 13), we included in this investigation the simultaneous measurement of dopamine and its neuronal metabolite DOPAC. Highly specific and precise GC/MS analysis was used throughout, allowing a direct comparison of diagnostic specificity and sensitivity for each analyte or analyte combination. In contrast to the other (non-N-methylated) catecholamines, the mass-spectral characteristics of the trifluoroacetyl derivatives of epinephrine are such that the molecules yield essentially only one fragment ion, derived from the ethylamine side chain (14). This ion is thus of low mass and questionable specificity in a

1 Nonstandard abbreviations: DHPG, 3,4-dihydroxyphenylglycol; NE, norepinephrine; GC/MS, gas chromatographic/mass-spectrometric; MEN 2a, multiple endocrine neoplasia type 2a; DOPAC, 3,4-dihydroxyphenylacetic acid; SIM, selected-ion monitoring; NICI, negative-ion chemical ionization; and EI, electron-impact ionization.
selected-ion monitoring (SIM) GC/MS assay. Under conditions of electron-capture negative-ion chemical ionization (NICI), however, the parent epinephrine molecule yields a prominent anion (15), providing an ideal basis for a specific SIM assay. By comparing electron-impact ionization (EI) and NICI SIM assays of epinephrine, we determined that the EI GC/MS assay reliably assessed increased epinephrine excretion in 24-h urine samples (16). In the present study we used the EI GC/MS assay for primary screening for increased epinephrine secretion; in samples where specific assignment of peaks was compromised by high sample background, we used NICI GC/MS.

**Materials and Methods**

**Patients**

Urine samples (all 24-h collections) from 2476 patients were assayed for this study. Patients were either referred directly to us by physicians for whom we provide a diagnostic service or urine samples were forwarded to us for analysis by GC/MS when prior biochemical testing by other techniques had proved inconclusive for diagnosing pheochromocytoma. Patients were classified as free of pheochromocytoma if either (a) initial biochemical testing by GC/MS catecholamine assay was negative and repeat testing was not requested by the referring physician or (b) initial biochemical screening was positive but repeat catecholamine assays by GC/MS in this laboratory and the clinical signs were negative. After excluding data from patients diagnosed with pheochromocytoma, we used the analytical data from the patients without tumors (n = 2430) to calculate the reference intervals (95% confidence intervals) for this investigation. Although one cannot be certain that a particular patient with negative biochemical assay results does not have a pheochromocytoma, we have been unable to find an example, in our database of >3000 patients, of a subject previously classified by the above criteria as not having a pheochromocytoma who later presented with a pheochromocytoma at any institution in New South Wales. Patients fitting either of the above criteria were assigned to our nontumor control group, a population we previously referred to as hypertensive (5). Although most of these patients presented with hypertension, some were investigated primarily for symptoms such as postural hypotension, headache, palpitations, and flushing. For this study we use the more general term nontumor to describe this group.

A diagnosis of pheochromocytoma was made in 46 patients. Histological confirmation of the diagnosis was obtained in 44 patients after surgical removal of a tumor. Of the remaining two patients classified as having a pheochromocytoma by positive biochemical screening plus the finding of adrenal masses on computed tomography scan, one is being managed conservatively because of poor general health and the absence of severe symptoms; the other declined surgery.

To ensure that the data for the nontumor group were not biased by results from a single patient undergoing multiple tests or samplings, only one value (the initial sample) from each patient was chosen for calculation of the reference intervals. In the pheochromocytoma group, several patients underwent multiple sampling after the initial positive test result; for those patients, we used the mean data from the pretreatment samples. Statistical analysis was performed by one-way analysis of variance followed by a post hoc Fischer PLSD test (Statview SE+ statistical package; Abacus Concepts Inc., Berkeley, CA).

**Assays**

Urine specimens were collected over HCl (6 mol/L; 10 mL/24-h sample) as a preservative. Samples (500 μL) of the urine specimens were taken for assay and, after incorporation of deuterated internal standards, alumina extraction, and derivatization with trifluoroacetic anhydride, we measured NE, DHPG, dopamine, and DOPAC concentrations by SIM GC/MS with a Hewlett-Packard (Palo Alto, CA) 5993A, 5970B, or 5987A GC/MS system, as described in detail elsewhere (5, 17). The assay of epinephrine was based on these same techniques, with EI GC/MS being used routinely. This was supplemented by NICI GC/MS to achieve optimal specificity in some urine specimens (~5%) when the sample background prevented accurate assessment of chromatogram peak areas in the EI mode and to confirm when methyldopa peaks were present in EI mode. SIM assays for derivatized epinephrine were based on the dominant fragment ion derived from the side chain (m/z 140) for the EI GC/MS assay and the molecular anion (m/z 567) for the NICI GC/MS assay. A deuterated internal standard, α,α,β-trideuterio-epinephrine free base (MSD Isotopes, Quebec, Canada), was incorporated to allow precise quantification. L-Epinephrine bitartrate for use as a reference sample was obtained from Sigma Chemical Co. (St. Louis, MO). Standard curves in both the EI and NICI modes were generated by taking a constant amount of the α,α,β-trideuterio-epinephrine standard and, for each point, adding serially reduced concentrations of nondeuterated epinephrine. After derivatization and GC/MS analysis, points on the standard curves were derived from single samples and single estimations of peak areas, and the curves were generated by unweighted least-squares linear-regression analysis. The standard curves (Figure 1) show the sensitivity (detection limit) of the assay for epinephrine to be 0.05 pmol (injected) and 0.2 pmol (injected) in NICI and EI assay modes, respectively. The interassay CVs for these assays ranged from 0.4% to 1.5% with standards of 12–100 pmol injected (n = 10 each).

Under the assay conditions reported here, capillary GC columns deteriorated rapidly (after only a few samples) and were not usable on a routine basis. Therefore, we used packed gas-chromatographic columns throughout. These glass columns, treated with dichlorodimethylsilane and packed with 3% OV-17 on Supelcoport 100–120 mesh (Supelco, Bellefonte, PA), provided stable chromatographic data (for >100 sample injections). Ul-

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tra-high-purity helium at a flow rate of 30 mL/min was used as carrier gas for both EI and NICI modes. In the NICI mode, methane was used as the CI reagent gas. For the 5987A GC/MS system, the GC injector port and GC/MS interface were maintained at 250 and 275 °C, respectively, and the source temperature was 100 °C. Perfluorotri-n-butylamine was used for calibrating the 5987A mass spectrometer by focusing on the ions at m/z 69, 219, and 502 in the EI mode and at m/z 452, 557, and 633 in the NICI mode. Ordinarily, the initial gas-chromatographic oven temperature of 130–140 °C was increased, after 1 min, to 220 °C at the rate of 30 °C/min.

Because of the highly specific assays used in this study, no drug or dietary restrictions were imposed on any of the patients. Methyldopa, a drug often prescribed for hypertension, is known to result in excretion of α-methyl catecholamine analogs that can interfere in fluorometric and HPLC-electrochemical detection assays for endogenous catecholamines (18, 19). Although α-methyl analogs do not interfere with the GC/MS assays used here for the catechols, methyldopa therapy is associated with reciprocally low concentrations of endogenous catechols because the catechols are replaced by α-methyl analogs in the 24-h urine samples from patients taking methyldopa (19); thus, methyldopa treatment may result in falsely normal urinary concentrations of endogenous catecholamines reported in the presence of pheochromocytoma. Because requests to our laboratory for sample analysis sometimes fail to state if patients are taking α-methyldopa, we routinely screen all samples for evidence of methyldopa administration (i.e., by simply scanning for the specific fragment masses corresponding to α-methyl analogs of the catecholamines during routine catecholamine GC/MS assays). Details of this procedure and its utility will be reported separately. When evidence of methyldopa therapy is found in a patient’s urine (this occurs in ~5% of samples), we advise the referring physician of the need to interpret the catecholamine excretion data with caution and, if clinical signs warrant, to collect another urine sample two weeks after cessation of methyldopa therapy.

Results
Calculation of Reference Intervals
As previously noted, urine NE and DHPG distributions in the non-tumor-bearing population are markedly skewed (5). We found this also to be the case for epinephrine, dopamine, and DOPAC. We used log-transformation to normalize the data; the distributions of the raw and log-transformed data are compared for epinephrine in Figure 2, which shows how closely the transformed data approach a normal distribution. The means and 95% confidence intervals for the non-tumor-bearing population (Table 1) are based on the logarithmic distributions. For all measures, an increased value was
defined as being above the 95% confidence interval, and this value was used as the upper reference limit for nonpheochromocytoma patients.

More than expected nonpheochromocytoma patients had an above-normal NE/DHPG ratio (98 patients, 4.0%), but only 17 patients (0.64%) were classified as falsely positive on the basis of a simultaneous increase of 24-h urinary NE and the NE/DHPG or urinary DHPG excretion—these being the criteria we previously suggested to improve diagnostic specificity (5). An expected false-positive rate of 26 of 1101 patients (2.4%) was observed for epinephrine data in the nonpheochromocytoma patients.

Pheochromocytoma Patients

Urinary NE excretion was above the 95% confidence limits for the nontumor group in 40 of the 46 patients with pheochromocytoma (i.e., 13% false-negative results if NE is the only diagnostic criterion). In the remaining six patients, epinephrine excretion was increased. Urinary epinephrine was above normal in 10 of the 19 patients with pheochromocytoma in whom epinephrine was measured (i.e., 47% false-negative results if epinephrine is the only diagnostic criterion). However, considering NE and epinephrine together as diagnostic criteria (i.e., if either NE or epinephrine is increased), provides a highly sensitive index (100%) of the presence of a pheochromocytoma. DHPG excretion above the reference range was seen in 19 of the 46 patients. The urinary NE/DHPG ratio was above the reference interval in 35 of the 46 pheochromocytoma patients.

In the pheochromocytoma patients, the mean and 95% confidence interval for urinary dopamine (calculated after log transformation) were 2.4 and 0.8–7.1 μmol/24 h; for DOPAC, these were 7.0 and 2.3–23.0 μmol/24 h. These data are not significantly different from, and have a high degree of overlap with, data from the nontumor population (Table 1).

Neither dopamine nor DOPAC was increased independently of the other catecholamines in any of the pheochromocytoma patients. Dopamine excretion was increased in 5 of 45 patients who had increases of norepinephrine or epinephrine (or both). DOPAC excretion was increased in only 2 of 44 patients with pheochromocytoma. Thus, in this series of patients, dopamine and DOPAC were insensitive diagnostic factors, and data for these analytes were not further analyzed.

The data we collected over the last two years of the investigation were from 1101 patients in whom all analytes, including epinephrine, were measured; these data included a subset of 19 patients with pheochromocytoma (out of the total of 46). Data from this latter set of patients were subdivided by whether NE or epinephrine excretion rates increased independently of each other. In 9 of 19 patients, NE excretion was increased but epinephrine was not; these patients were classified as NE-secreting. In 6 of 19 patients, epinephrine excretion was increased but NE was normal; these patients were classified as epinephrine-secreting. The remaining four patients exhibited marked increases of both NE and epinephrine excretion and were classified as mixed NE/epinephrine-secreting. The major differences between these subgroups can be seen by comparing the mean analytical data for NE, epinephrine, and DHPG with data from the nonpheochromocytoma group (Figure 3). As shown, DHPG excretion was significantly greater for each of the tumor classification subgroups than for the nontumor patients. Further comparison of DHPG with epinephrine secretion in these groups showed that DHPG was not significantly correlated with epinephrine in the NE secretors ($r^2 = 0.087, P = 0.4$) but was
significantly correlated with epinephrine in the mixed NE/epinephrine secretors ($r^2 = 0.84, P = 0.0002$) and in the epinephrine secretors ($r^2 = 0.46, P < 0.0014$).

**Diagnostic Potential of Analytes**

On the basis of the data obtained from both the nontumor and pheochromocytoma patient groups, we calculated the overall diagnostic sensitivity, specificity, and predictive power of the analytes (21). As shown in Table 2, the individual diagnostic sensitivities of urinary NE and epinephrine are reduced by the presence of uniquely NE- or epinephrine-secreting tumors but, as noted above, an increase of either urinary NE or epinephrine is 100% sensitive in the diagnosis of all pheochromocytomas.

**Discussion**

In this investigation 46 pheochromocytomas were diagnosed in a population of 2476 referred patients. This rate, 1.8%, is nearly 20-fold more than that suggested in the literature for the hypertensive population at large (4, 22, 23). However, this rate of detection has been fairly constant in our laboratory for more than six years. From 1985 to 1988, we identified 27 pheochromocytomas in a population of 1401 hypertensive patients (1.9%). In the last two years of the current investigation (1988-1990), pheochromocytoma was diagnosed in 19 of 1101 referred patients (1.7%). As a tertiary referral laboratory, we acquired a predominantly (~75%) hypertensive patient group that had been screened by physicians who referred the patients to us with a provisional diagnosis of possible pheochromocytoma. This factor may contribute substantially to our detecting a higher proportion of pheochromocytomas in our patients than might be predicted from the literature.

In the 19 pheochromocytoma patients in whom urinary epinephrine excretion as well as NE excretion was measured, 10 patients produced excessive amounts of epinephrine; 4 of these patients produced increased amounts of both NE and epinephrine, but in 6 patients (i.e., >30%) only epinephrine excretion was increased. These findings agree with a report showing 5 of 14 pheochromocytoma patients to excrete epinephrine exclusively (10). However, although the data of that other study may have been biased [four of the five pheochromocytoma patients had an abnormal endocrine history (one acromegaly and three presumed MEN 2a) (10)], in

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**Table 2. Diagnostic Sensitivity, Specificity, and Predictive Power for Urinary NE, Epinephrine, and DHPG and the NE/DHPG Ratio**

<table>
<thead>
<tr>
<th>Urinary analyte</th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
<th>Predictive power, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE</td>
<td>88.5</td>
<td>99.3</td>
<td>52.9</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>67.9</td>
<td>97.7</td>
<td>45.2</td>
</tr>
<tr>
<td>NE or epinephrine</td>
<td>100</td>
<td>97.4</td>
<td>41.8</td>
</tr>
<tr>
<td>DHPG</td>
<td>63.0</td>
<td>99.2</td>
<td>70.8</td>
</tr>
<tr>
<td>NE/DHPG</td>
<td>80.7</td>
<td>96.1</td>
<td>31.9</td>
</tr>
</tbody>
</table>
the present investigation only one patient had familial pheochromocytoma (MEN 2a; established during this study) and none had coexisting endocrinopathies.

Apart from the patient with MEN 2a who was detected during a screen for that disease, the remaining five patients reported here with uniquely epinephrine-secreting pheochromocytomas had symptoms typical of pheochromocytoma but did not present primarily with hypertension; one was hypertensive and, notably, exhibited the greatest increase of epinephrine for this group (mean, 1554 nmol/24 h). These data agree with recent reports that hypertension is not manifest in patients harboring epinephrine-secreting tumors (10, 11). Also important in this context is a report of six normotensive patients who presented with sudden unexplained pulmonary edema and heart failure secondary to cardiomyopathy induced by previously undiagnosed pheochromocytomas (24). Urinary excretion of vanillylmandelic acid was normal in two of these patients for whom a diagnosis of pheochromocytoma had been entertained (24), but epinephrine production was not measured in any of the subjects.

If our present data on the relative frequency of epinephrine-secreting pheochromocytomas can be related to our earlier patient population (n = 1374) subset in whose samples epinephrine was not measured but 27 NE-secreting pheochromocytomas were identified, perhaps some uniquely epinephrine-secreting tumors may have been present in the earlier subset but not detected. If this was the case, our earlier calculations of sensitivity would have systematically overestimated the sensitivity of NE in the diagnosis of pheochromocytomas.

Both epinephrine and NE (or, possibly, metanephrine and normetanephrine) must be assayed separately to ensure that both NE- and epinephrine-secreting pheochromocytomas are found. Despite having access to HPLC, some laboratories are currently measuring and reporting only total urinary catecholamine excretion. We now regard the assay of total catecholamines in urine, as opposed to assays of NE and epinephrine individually, to be diagnostically inadequate, because the epinephrine-excretion rates in some uniquely epinephrine-secreting tumors might be insufficient to noticeably increase the concentration of total catecholamines. Indeed, in this study one epinephrine-secreting pheochromocytoma patient did not have an increased excretion of total catecholamines (assessed by the arithmetic sum of NE and epinephrine measurements). For the same reasons, we argue against the use of total urine metanephrines or urine vanillylmandelic acid in pheochromocytoma diagnosis.

Comparison of the mean data for the nontumor population and the pheochromocytoma subgroups (see Figure 3) is informative. Besides delineating the unique NE- and epinephrine-secreting populations, the data show DHPG excretion to be significantly increased in each tumor subgroup (Figure 3). Although this factor contributes to a poor diagnostic sensitivity of the NE/DHPG ratio in epinephrine-secreting pheochromocytomas, it indicates that increased DHPG and epinephrine excretion are associated. Under normal circumstances, DHPG is a product of the action of neuronal monoamine oxidase on NE after neuronal reuptake of the amine. In comparison, epinephrine is not a good substrate for peripheral neuronal uptake sites (25). Furthermore, exogenously administered NE does not lead to the production of significant amounts of DHPG (6), making it unlikely that the DHPG originates from peripheral uptake of epinephrine. More probably, the DHPG is formed within the tumor or, alternatively, is derived from increased sympathoadrenal activity as a result of the disease state. Despite the significant correlation between DHPG and epinephrine secretion in the epinephrine-secreting subgroups, the DHPG concentration was not a good predictor of urinary epinephrine concentration in the epinephrine-secreting group (r² = 0.46).

One consequence of the increased production of DHPG in association with unique epinephrine-secreting pheochromocytomas is that measuring the DHPG concentration, in addition to epinephrine, reduces the false-positive rate from 2.3% to 0.6%. This is similar to the effect of using DHPG in addition to NE in NE-secreting pheochromocytomas in this and our previous investigations (5). In the 40 NE-secreting tumor patients (i.e., NE alone and NE/epinephrine mixed) reported here, the combined use of NE, DHPG, and the NE/DHPG ratio reduced the rate of false-positive diagnosis from ~2% to 0.4% without reducing sensitivity.

For the patients in whom all analytes were assayed, the urinary NE/DHPG ratio was above normal in eight of nine pheochromocytoma patients who secreted NE exclusively and was normal in all patients who secreted epinephrine exclusively. Thus the hypothesis that this ratio can be used to distinguish between neuronal and pheochromocytoma origin of NE (6, 26) is supported only as long as it is applied to patients thought to have exclusively NE-secreting tumors.

Measurement of the urinary excretion of dopamine or its metabolite DOPAC appears to provide no additional help in diagnosing pheochromocytoma. Although some have proposed that measuring dopamine excretion may be of prognostic benefit in malignant pheochromocytoma (12, 13), our data do not support this: three of our patients with proven malignant pheochromocytoma had dopamine and DOPAC concentrations within normal limits.

Our data indicate a need to revise the traditional views that most pheochromocytomas secrete NE predominantly and that uniquely epinephrine-secreting tumors are rare (4, 11, 23). Currently, essentially the hypertensive population is recommended to be screened for pheochromocytoma (4); however, in light of our data and the reports noted above, failure to exhibit hypertension cannot be considered grounds for excluding the diagnosis of pheochromocytoma. Similarly, measuring NE production alone, or total catecholamines, would be insufficient in screening for pheochromocytoma in normotensive and hypertensive patients who might have

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uniquely epinephrine-secreting tumors. We suggest that pheochromocytoma will remain biochemically undiagnosed in many patients, with possibly fatal consequences, unless specific assays for each catecholamine are used.

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