Measurement of catecholamines and their metabolites in urine

C Weinkove

**Introduction**

The catecholamines of clinical interest, noradrenaline, adrenaline, and dopamine, are simple dihydroxyphenylamines which differ in their side chain structure. Dopamine is formed by the decarboxylation of dihydroxyphenylalanine (DOPA) and is in turn hydroxylated to produce noradrenaline, which is then methylated to produce adrenaline. All three are important central nervous system neurotransmitters, with adrenaline, produced from the adrenal medulla, having an additional role as a circulating hormone.

As well as sharing a common synthetic pathway they are all degraded by a mixture of side chain oxidation and O-methylation of the hydroxyl groups on the ring. If metabolism is complete this will produce 4-hydroxy-3-methoxy mandelic acid (HMMA), commonly called vanillyl mandelic acid (VMA), from both noradrenaline and adrenaline, and homovanillic acid (HVA), from dopamine. If O-methylation of the parent compound alone occurs, this will yield normetadrenaline, metadrenaline, and 3-methoxytyramine from noradrenaline, adrenaline, and dopamine, respectively. The catecholamines and some of their O-methylated derivatives are excreted as glucuronide and sulphate conjugates in the urine.

Increased urinary excretion of unconjugated catecholamines (noradrenaline, adrenaline, and dopamine) or their main metabolites, HMMA, HVA, and the methoxycatecholamines (metadrenaline and normetadrenaline) have been used as a tumour marker for neuroblastoma in children and phaeochromocytoma in adults. Both these tumours arise from primitive neural crest tissue and have the capacity to secrete catecholamines directly into the circulation. Increased urinary catecholamine excretion and high plasma catecholamine concentrations have also been used to monitor "stress" under different environmental conditions.

**Phaeochromocytoma**

There are few clinical conditions in which the responsibility for diagnosis depends more on the laboratory than phaeochromocytoma. This is a rare catecholamine-secreting tumour of neural crest origin that characteristically produces episodes of hypertension, severe headaches, and sweating. Patients with phaeochromocytoma more commonly have sustained rather than intermittent hypertension. Less commonly they may present with one or more of the following: loss of consciousness; and chest or abdominal pain, or both. Undiagnosed, the excess circulating catecholamines may produce cardiac muscle necrosis, with sudden death precipitated by surgery or pregnancy remaining a common mode of presentation.

To add to our embarrassment 90% of these histologically benign tumours can be removed and do not recur after surgery. Unfortunately, most clinicians limit investigation to young patients with intermittent or uncontrollable hypertension. In fact phaeochromocytomas have been found in subjects from 9 months to 90 years of age and are more common in older (50 years and over) than younger subjects.

The detection of this rare disease is not helped by laboratories having to rely on out-moded and uncontrolled analytical methods. Clinicians should be encouraged to look for this curable disorder and not be put off by unrealistic demands that all medication be discontinued and stringent dietary restrictions enforced. Once considered, the diagnosis of phaeochromocytoma could be made by proving increased release of the catecholamines noradrenaline and adrenaline into the circulation and their excretion with their metabolites in the urine.

Plasma catecholamine concentrations can now be more easily measured. I would recommend that free catecholamines be measured in 24 hour urine collections for the following reasons: (i) they are more stable in acid urine than plasma; (ii) catecholamines are present in higher concentrations in the urine and are therefore easier to measure than in plasma, which must be rapidly separated, frozen, and stored at −20°C; and (iii) plasma catecholamine concentrations provide single point readings which vary with the immediate physiological and pathological condition of the patient, whereas urinary catecholamine excretion provides an integrated measurement.
of catecholamine release and is theoretically more valid.

Measurement of plasma catecholamine concentration does have a place in the following circumstances: (i) in the anuric patient; (ii) when there is difficulty in obtaining urine specimens—for example, in some children or very uncooperative patients; (iii) when drugs or other substances interfere with urine assays; and (iv) rarely, to help localise a pheochromocytoma.

As well as the parent compounds, noradrenaline, adrenaline, and dopamine, there are several catecholamine metabolites which can be measured in the urine. These include metadrenaline and normetadrenaline, often combined in one assay and referred to as total metadrenalines. HMMA was the most commonly used analyte in a United Kingdom survey done for the Catecholamine External Quality Assessment Scheme, though experience has shown this analyte to be less reliable than metadrenalines. HVA, an end product of dopamine metabolism, will theoretically be more appropriate for the diagnosis of neuroblastoma than pheochromocytoma.

Experience in running an external urinary catecholamine quality assessment scheme has highlighted many deficiencies in laboratory performance. These can be attributed to: (i) the slavish acceptance of previously reported recoveries; (ii) the failure to put primary standards through the same analytical process as patients' samples; (iii) the infrequency with which the assay is carried out; (iv) the rarity of positive results and the delegation of the assay to less experienced laboratory staff.

While most pheochromocytomas (about 90%) occur in the adrenal glands, they may be found in nests of sympathetic nervous tissue anywhere from the neck to the groin. Adrenal tumours may be identified by computed tomography and ultrasound examination. The combination of high catecholamine excretion, radiological signs of an adrenal tumour, and appropriate symptoms may reasonably lead to the suspicion that the adrenal tumour is a pheochromocytoma. This is not always the case. Adrenal cortical adenomas are common. To confirm that the adrenal tumour is the true and only site of catecholamine production (10% of pheochromocytomas are multiple), a new radiolabelled material, metaiodobenzylguanidine (MIBG), should be used. This compound binds specifically to catecholamine-containing tissue and persists longer within a pheochromocytoma than other tissues containing less catecholamine. MIBG is increasingly being used as a non-invasive method of locating the aberrant pheochromocytoma. As with all localisation techniques, both false negative and false positive results have been reported with MIBG scans.

Neuroblastoma

Neuroblastoma is the most common malignant soft tissue tumour of childhood, usually presenting before the age of 3. These tumours originate from ectodermal neuroblasts and, like pheochromocytomas, are frequently found adjacent to or within sympathetic ganglia. Unlike pheochromocytomas, however, they commonly present as an asymptomatic mass causing local pressure symptoms with metastases at the time of diagnosis.

The excretion of catecholamines and their metabolites, although invaluable in the diagnosis of neuroblastoma, are less sensitive as biochemical markers of this tumour than for pheochromocytoma. The Royal Manchester Children's Hospital currently measures dopamine, HVA, and HMMA in the urine of all suspected cases of neuroblastoma, using high power liquid chromatography. They have found that HVA and HMMA will diagnose more than 95% of cases, but they discovered one patient in whom only dopamine was unequivocally raised.

Methods

I describe two assay methods, which, together, should cover the diagnoses of both pheochromocytomas and neuroblastomas. Both use high power liquid chromatography (HPLC) techniques. Experience has shown that HPLC is more specific, accurate, and sensitive than conventional chemical and similar chromatographic methods for the measurement of catecholamines and their metabolites in urine. I have little personal experience of the latter methods and cannot recommend them until more is known about their performance in External Quality Control Schemes. Many otherwise good laboratory methods place restrictions on the patient's diet and treatment, making them unacceptable to the clinician.

The HPLC methods described in this paper can, and should, be automated. This improves assay reliability because, as well as handling larger sample numbers, an adequate number of standards and quality control samples can be run with each batch without extra effort. With an automated method the laboratory could encourage the clinician to send urine specimens on all symptomatic hypertensive patients. Those laboratories with a small workload un-
able to justify the expense of the essential equipment or lacking the experienced staff for these techniques, should consider sending their specimens to an appropriate laboratory for analysis.

The first method uses a simple initial alumina extraction but depends for specificity on HPLC separation and sensitive serial electrochemical detectors. Our laboratory is in the process of adopting a new commercially available sample preparation method (ASTED) which will be covered briefly.

With urinary noradrenaline excretion used as a marker to identify the patient with phaeochromocytoma and urinary dopamine excretion used to recognise children with neuroblastomas, the measurement of urinary free catecholamine excretion using HPLC would, theoretically, provide a complete service. Because the available biochemical markers of neuroblastoma lack sensitivity, I have included HPLC methods for the measurement of urinary HVA and HMMA excretion. This would identify those neuroblastomas excreting predominantly catecholamine metabolites.

Measurement of urinary free catecholamines using HPLC with electrochemical detection

**PRINCIPLE**

Many HPLC methods have been described for the measurement of urinary catecholamines. The following procedure is used to measure noradrenaline, adrenaline, and dopamine in both urine and plasma. Catecholamines are adsorbed from the urine with activated alumina, eluted with acid, separated by reverse phase ion-pair chromatography and measured by electrochemical detection. Ascorbic acid is used as an antioxidant to prevent catecholamine degradation\(^8\) while an autoinjector permits unattended batch analysis of many samples.

Measurement is based on the ability of catecholamines to be reversibly oxidised to their corresponding o-quinones. In the method to be described three electrodes, set at different potentials and arranged in series, cause sequential oxidation and reduction which screens out interfering compounds. Less selective detection systems will require more extensive sample preparation.

**COLLECTION OF URINE SPECIMENS**

Collect a 24 hour specimen of urine into a bottle containing 50 ml of 500 mmol/l sulphuric acid (or 10 ml concentrated HCl) and measure the volume. Store an aliquot at 4°C or at −20°C if analysis is delayed for some months. I have found that, provided the specimens are acidified (pH < 2), they keep for months and may be transported by ordinary post to other centres for analysis. (Plasma catecholamines are more labile and samples must be kept frozen until analysed).

**REAGENTS**

All reagents are Analar grade from BDH unless otherwise indicated.

1. **Extraction buffer, 1 mol/l TRIS buffer (pH 8.6) with 2% EDTA:** Dissolve 36-62 g Trizma HCl (Sigma), 93-05 g Trizma base (Sigma), and 20 g of the disodium salt of EDTA in 700 ml of distilled water. Adjust the pH to 8.6 with concentrated HCl and make up the final volume to 1 litre with distilled water.

2. **Hydrochloric acid:** 0-02 mol/l.

3. **Acetic acid:** 0-1 mol/l.

4. **Mobile phase:** 0-1 mol/l citrate/phosphate buffer (pH 3-2), containing 3-8 mmol/l 1-heptanesulfonic acid (the ion-pair re-agent), 0-17 mmol/l EDTA, 9-5% methanol (v/v), 3-5% acetonitrile (v/v).

Dissolve 15-8 g of citric acid (monohydrate), 7-02 g of Na\(_2\)HPO\(_4\) (anhydrous), 0-074 g disodium EDTA and 0-880 g 1-heptanesulfonic acid (sodium salt, Sigma) in 700 ml of distilled water. Check that the pH is 3-2 and make the final volume up to 1 litre with distilled water. Add 110 ml of methanol and 40 ml of acetonitrile (both HPLC grade) and mix. Before use, the mobile phase must be filtered and degassed under vacuum using a 0-2 μm membrane.

5. **Stock standard solutions:** 1 mmol/l of noradrenaline, adrenaline, dopamine and 3,4-dihydroxybenzylamine (DHBA, the internal standard) are prepared in 0-02 mol/l HCl.

Individual stock standard solutions are prepared by separately dissolving each of the following in 0-02 mol/l HCl and then making their respective volumes up to 100 ml with 0-02 mol/l HCl: 33-7 mg norepinephrine bitartrate (monohydrate) (“noradrenaline”; Sigma); 33-3 mg epinephrine bitartrate (“adrenaline”; Sigma); 18-9 mg 3-dydroxytyramine (“dopamine”; Sigma); 22-0 mg 3,4-dihydroxybenzylamine (Sigma). These solutions may be stored at 4°C for up to six months.

6. **Intermediate standards:** 20 μmol/l are prepared by diluting 2 ml of each stock standard individually to 100 ml with 0-02 mol/l HCl. These can be stored for up to three months at 4°C.

7. **Working standards:** prepared from the intermediate standards immediately before each assay and not reused (unless stored at −20°C). Noradrenaline (1 ml), 0-4 μmol of adrenaline, and 5 μl of dopamine intermediate standards are combined and made up to 50 ml with 0-1 mol/l acetic acid. This provides a top standard with 0-4, 0-16, and 2-0 μmol/l of noradrenaline, adrenaline, and dopamine, respectively. Three more standards are prepared by doubling dilution of the top standard with 0-1 mol/l acetic acid. A zero standard or “blank” consists of 0-1 mol/l acetic acid alone.

8. **Working internal standard:** made by preparing a 1 in 200 dilution of the intermediate DHBA (see below) in 0-02 mol/l HCl. This can be stored at 4°C for up to six weeks.

9. **Ascorbic acid:** 2-83 mmol/l is freshly prepared by dissolving 50 mg in 100 ml of water.

10. **Activated alumina:** prepared as previously described.

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APPARATUS
(1) Spherisorb ÖDS-2 5 µm analytical column 250 × 4.6 mm.
(2) ESA Coulorchem electrochemical detector Model 5100 A with conditioning cell Model 5021 (conditioning cell set at +0.35 V) and analytical cell Model 5011 (detector 1 set at +0.05 V, detector 2 set at −0.35 V).
(3) Pye Unicam CDP 4 computing integrator.
(4) Multi-tube vortexer (SMI).

PROCEDURE
During the extraction procedure described below, all test tubes, reagents, and samples should be kept at 4°C on a cold tray. To 4 ml poly styrene tubes (Sartstedt) add: (1) 1.5 ml distilled water; (2) 0.1 ml ascorbic acid solution; (3) 0.2 ml 100 mmol/l DHBA in 0.02 mol/l HCl; (4) 25 µl of sample or standard; (5) 50 mg alumina; (6) 0.5 ml of extraction buffer. Cover the rack of test tubes with clingfilm and vortex for 10 minutes. Centrifuge at 2500 rpm at 4°C for 30 seconds and aspirate the supernatant. Wash the alumina with 2 ml distilled water. Vortex for 20 seconds, centrifuge at 2500 rpm for 30 seconds at 4°C, and aspirate the supernatant. After repeating this water wash add 250 µl 0.1 mol/l acetic acid (previously passed through a 0.2 µm filter) to the alumina. Vortex for 120 seconds. Centrifuge at 2500 rpm at 4°C for 10 minutes. Carefully removed acid eluate and inject on to HPLC column.

CHROMATOGRAPHY
Set the mobile flow rate at 1.2 ml/minute and monitor the column effluent at −0.35 volts (detector 2) using a 0–200 nA sensitivity range and response time setting of 10 seconds. The retention times for noradrenaline, adrenaline, internal standard and dopamine are 4.2, 5.1, 6.1 and 8.1 minutes, respectively. The run time per sample is 12 minutes. Measure the relevant peak heights.

CALCULATION
The ratio of peak heights of the catecholamines to the peak heights of the internal standard (DHBA) is linear over a large range (10–2000 nmol/l). The four standards and blank are used to calculate the concentration of catecholamines in the unknowns by linear regression. Pathological specimens with high catecholamine concentrations are diluted 10-fold to bring them within the range of the standards.

INTERPRETATION
In a personal series of 150 hypertensive patients who did not have phaeochromocytoma all had total free catecholamines (noradrenaline + adrenaline) of less than 2.0 µmol/24 hours. Most excreted less than 0.6 µmol/24 hours, with only one producing more than 1.3 µmol/24 hours (1.8 µmol/24 hours). Seven of eight patients known to have phaeochromocytoma had excretion rates between 2.0 and 6.0 µmol/24 hours. One produced 1.8 µmol in 24 hours.

I had had occasional false positive results, as shown by a failure to find a phaeochromocytoma on repeated intensive investigation or when repeat assays showed normal urine catecholamine excretion. Obviously false positive tests include the administration of catecholamines or dobutamine to ill patients. Most phaeochromocytomas secrete noradrenaline. The findings of high urinary adrenaline excretion with normal urinary noradrenaline excretion should lead you to suspect some other cause, such as severe “stress” or a neurological disorder. One patient with persistently raised urinary adrenaline excretion was found at necropsy to have an infiltrative malignancy of his brain and spinal cord, and no phaeochromocytoma. Nevertheless, the presence of persistently raised urinary catecholamine excretion (adrenaline or noradrenaline) does justify minimum investigation such as adrenal ultrasound examination, computed tomography scan, and an MIBG scan.

If biochemical tests remain positive and attempts to localise the tumour fail catecholamine release as a result of stress may be suspected. A reduction in catecholamine excretion induced by sleep or clonidine, or both, would support this diagnosis. In practice this has not been a major diagnostic problem. Failure to localise a tumour precludes surgery anyway and the patient must be managed medically before repeating radiographic localisation.

I have heard of only one false negative, urine free catecholamine excretion result (using HPLC and electrochemical detection) in an adult with a phaeochromocytoma (Sinclair D, Shenkin A, personal communication). Unfortunately, urine metabolites were not measured.

Interpretation of urine catecholamines and their metabolite excretion in children is difficult, because of physiological variation of excretion rate with age and because it is not always possible to obtain 24 hours urine collections. The biochemical findings in 174 patients with neuroblastoma have been reported. Simple assays for dopamine alone were not generally available throughout the 16 year period required to collect this large number of cases. These workers found that total catecholamines (the sum of adrenaline, noradrenaline, and dopamine) were marginally more sensitive (84%) than HMMA (78%) in diagnosing neuroblastomas, with virtually identical specificities. They provide an age related reference range for children, expressing results per mol of creatinine, for the total catecholamines, total metadrenalines, and HMMA. The combination of total catecholamine and HMMA excretion increased the sensitivity to 98.9%. It is important to know whether dopamine excretion alone would be a better discriminator of neuroblastomas than total catecholine excretion.

Our laboratory (unpublished data) has measured the individual catecholamine excretion per mole of creatinine in 46 paediatric inpatients, 482 healthy school children (aged 5–16), and 17 outpatients under the age of 5. Results (table) confirm that catecholamine excretion rates fall from high values under the
Measurement of catecholamines and their metabolites in urine

Ratio of urinary free catecholamine to creatinine concentrations at different ages in children without phasechromocytomas or neuroblastomas

<table>
<thead>
<tr>
<th>No of children</th>
<th>Age (years)</th>
<th>Noradrenaline/Creatinine (µmol/mol) Mean</th>
<th>Mean + 2SD</th>
<th>Adrenaline/Creatinine (µmol/mol) Mean</th>
<th>Mean + 2SD</th>
<th>Dopamine/Creatinine (µmol/mol) Mean</th>
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Age of 5 years to approach adult values by the age of 10. None of these children had a phaeochromocytoma and only five had a neuroblastoma. There was an overlap in both urine noradrenaline and adrenaline excretion between unaffected children and those with neuroblastoma. Urine dopamine excretion, however, was a better discriminator between the "normal" and our small series of patients with neuroblastomas. The highest dopamine excretion rate in an unaffected child was 1500 µmol/mol creatinine, well below the lowest dopamine excretion rate of 5000 µmol/mol creatinine in one of the children with a neuroblastoma. A larger series may blur this encouraging difference.

I have had few problems with the above assay despite the broad range of medication our patients receive, such as paracetamol, Aldomet, and labetalol which are known to interfere with catecholamine assays.12

The ASTED modification for the measurement of urinary free catecholamine

PRINCIPLE

In more than 95% of urine samples we process sample preparation with a simple alumina extraction is sufficient to avoid assay interference. Specificity is preserved by the sophisticated HPLC separation, supported by serial electrochemical oxidation and reduction described above. Interfering peaks found in the occasional urine specimen have been managed by combining the alumina extraction with a second solid phase extraction or measuring the patient’s plasma catecholamine concentration.

To further reduce interference and simplify the urinary catecholamine assay we are testing the ASTED system. This method has been published13 and is commercially available as an ASTED unit from Gilson Medical Electronics (Villiers-le-Bel, France). ASTED is the acronym for “automated sequential trace enrichment of dialysate,” an automated sample preparation technique. It involves dialysis of the sample and concentration of the analyte on a suitably packed stainless steel trace enrichment cartridge (TEC) which replaces the loop on the Rheodyne injection valve.

For the catecholamine assay, the urine samples are not dialysed but are simply concen-

tered on the packing material, 10 µm diameter Separon S-hydroxyethylmethacrylate-BIO 1000 sulphobutyl (HEMA-SB), obtained from Anachem Ltd (Luton, Bedfordshire). A washing phase removes interfering compounds from the TEC before the sample is flushed on to the column.

The sample preparation has the added advantage of allowing the measurement of urinary metadrenaline and normetadrenaline in the same run as the urinary free catecholamines. While reducing the rate of sample throughput, automated sample preparation substantially enhances assay precision and specificity. This would reduce the need for a very sophisticated electrochemical detector, such as the one we use. Furthermore, once established, the assay can be reliably performed by less experienced technical staff.

APPARATUS AND PROCEDURE

This information has already been published13 and is fully covered in the application document (No 7) provided by Gilson Medical Electronics. Initial problems with the system were found to be due to inadequate packing of the TEC. It is important to obtain a reliable source of these cartridges or to learn to pack your own.

INTERPRETATION

This is identical to that provided above, with the added advantages of the simultaneous measurement of noradrenaline and metadrenaline and a reduction in analytical interference by drugs or other catecholamine-like compounds.

Simultaneous assay for urinary 4-hydroxy-3-methoxymandelic acid (HMMA), 5-hydroxyindole acetic acid (5-HIA), and HVA by isocratic HPLC with electrochemical detection

This method is described by Davidson,14 with some modifications (personal communication).

PRINCIPLE

Acidified urine is extracted with ethyl acetate, which contains an internal standard. There is rapid uptake of the acidic metabolites of the biogenic amines, which are back extracted into
phosphate buffer (pH 7.0), and the ethyl acetate is removed. The acids are then separated and measured using HPLC with electrochemical detection. Samples are run in duplicate with and without added standards ("spiked" and "unspiked"). This corrects for any matrix effects and helps to avoid misidentifying peaks caused by drugs or their metabolites.

Though of no value in the diagnosis of phaeochromocytoma or neuroblastoma, measurement of urinary 5-hydroxyindole acetic acid is useful in the diagnosis of the carcinoid syndrome which shares some clinical features with phaeochromocytoma.

COLLECTION OF URINE SPECIMENS
A twenty four hour urine collection must be made into opaque bottles containing 10 ml concentrated HCl.

REAGENTS
1 Stock combined HMMA, 5-HIAA, and HVA standard (1-1 mmol/l)
Weight out 218 mg VMA (Sigma No H 0131), 210-3 mg 5-HIAA (Sigma No H 8876), and 200-4 mg HVA (Sigma No H 1252). Dissolve and make up to 1000 ml with 0-16 mol/l (10 g/l) boric acid. Stored at 4°C this is stable for about two months.
2 Stock isovanillic acid (IsoVA) (Internal standard)
Weigh out 100 mg of isovanillic acid (Sigma No 1 5878) and dissolve in 100 ml ethanol.
3 Working internal standard
To each 10 ml of ethyl acetate (BDH No 10108) add 0-2 ml of stock isovanillic acid.
4 Mobile phase
(i) Phosphoric acid (2 mol/l)
Carefully add with mixing, 136 ml of concentrated orthophosphoric acid (BDH ARISTAR No 10173) to about 800 ml water. Then make up to 1000 ml with water.
(ii) Methanol (Rathburn Chemicals Ltd, Walkerburn, Scotland) HPLC grade (No RH 1019)
(iii) Working mobile phase
Weigh out 10-9 g KH₂PO₄ (BDH No 10203) and 100 mg EDTA (BDH No 10093). Dissolve in water and add 160 ml methanol and 10 ml of 2 mol/l phosphoric acid. Make up to 1000 ml with water. This solution should be filtered (0-45 μm) and degassed before use.
5 Hydrochloric acid (1 mol/l)
Carefully add with mixing, 87-3 ml concentrated HCl (BDH No 10125) to water in a 1 litre volumetric flask. Make up to 1000 ml with water.
6 Phosphate buffer (pH 7-0)
(i) Solution A: KH₂PO₄ (0-07 mol/l)
Weigh out 9-073 g KH₂PO₄ (BDH No 10203) and dissolve in 1000 ml water.
(ii) Solution (B) Na₂HPO₄. 2H₂O (0-07 mol/l)
Weigh out 11-87 g Na₂HPO₄. 2H₂O (BDH No 3 0157) and dissolve in 1000 ml water.
(iii) Working phosphate buffer
Transfer 41.3 ml of solution (A) to a 100 ml volumetric flask and make up to 100 ml with solution (B).

APPARATUS
The HPLC system consisted of a Waters Model 6000 A solvent delivery system and a U6K injector. The electrochemical detector was a TL-5 glassy carbon electrode obtained from Bioanalytical Systems Inc, West Lafayette, Indiana 47906, USA. Separation was achieved on a 150 × 4.6 mm ODS 5 μm particle size column (Bio Rad Laboratories Ltd, Watford, Hertfordshire, England). The detector output was monitored on a Philips PM 8252 pen recorder.

PROCEDURE
Transfer 1-0 ml of each urine into test tubes, add 100 μl of stock combined standard, and vortex mix. This is the "spiked urine".
Put 200 μl of urine, alternatively "spiked" or "unspiked", into conical tubes and to each add 20 μl of 1 mol/l HCl and 1-0 ml of working internal standard solution. Vortex mix each tube for 15 seconds and centrifuge. Transfer 200 μl of the upper organic layer to fresh conical, glass stoppered tubes containing 1-0 ml of phosphate buffer. Vortex mix each tube for 15 seconds and centrifuge. Remove and discard the upper ethyl acetate layer and immediately add 20 μl of 1 mol/l HCl to the aqueous phase. Mix and inject 25 μl on to the column.

CHROMATOGRAPHY
Set the mobile flow rate at 1-0 ml/minute and monitor the column effluent at +0-67 volts using a 0-100 nA sensitivity range and filter setting of 0-5 second. The retention times for HMMA, 5-HIAA, HVA and IsoVA (internal standard) are about 2-5, 10, 15 and 17-5 minutes, respectively. Measure the relevant peak heights.

CALCULATION
Excretion of metabolite = U × 100 x V/(S - 10/11 × U) μmol/24 hours where U and S are the analyte to internal standard peak height ratios of the "unspiked" and "spiked" samples, respectively, and V is the 24 hour urine volume in litres.

INTERPRETATION
The adult reference range for 24 hour urine excretion of HMMA, 5-HIAA, and HVA are <35, <50, and <40 μmol/24 hours, respectively. As with the measurement of urinary free catecholamines, major errors are caused by unacidified urine and inaccurate urine collection. Discard all urine specimens with a pH of more than 4-0. Urine creatinine should be measured to assess the completeness of the urine collection.

The finding of increased urine catecholamine metabolite excretion makes it imperative that the clinician search for a phaeochromocytoma or neuroblastoma. If there is any doubt about the validity of the assay it should be repeated on a fresh urine collection made after all drugs have been discontinued.

I am indebted to our laboratory staff, particularly Anne Grennan and Paul Reed, in establishing and validating our own urine free catecholamine assay. I thank Messrs DF Davidson, Green Cooper and Turnell for permission to publish their methods and
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for additional details of their own urine assays. I am grateful to Dr GM Addison and the biochemistry staff at Royal Manchester Children's Hospital for information on the biochemical diagnosis of neuroblastoma.

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