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Short Communication

Analysis of urinary and plasma catecholamines using a single LC–EC system*

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Introduction

In the investigation of catecholamine-secreting tumours (e.g. phaeochromocytomas) demonstration of elevated tumour-derived catecholamine concentrations is the mainstay of the clinical–biochemical diagnosis. The level of catecholamine secretion may be assessed indirectly by measurement of metabolites in urine or directly as plasma or urinary catecholamines [1]. Measurement of basal concentrations of plasma catecholamines has been suggested [2] as being a better indicator of hypersecretion of catecholamines than measurement of the urinary metabolites. Failure to suppress elevated plasma catecholamine levels with a ganglion-blocking drug, e.g. pentolinium, is diagnostic of the presence of a phaeochromocytoma. Measurements of plasma catecholamines are useful in localization of catecholamine-secreting tumours through selective venous sampling via a catheter [3].

Routine screening tests have been based mainly on measurement of urinary catecholamine metabolites (e.g. VMA or metanephrines) which require different analytical methodologies and have a high incidence of false-negative results [4]. Screening for phaeochromocytoma by measurement of urinary free noradrenaline has a reported 100% sensitivity (i.e. no false negatives [5]) and is especially useful in detection of paroxysmally-secreting tumours where elevated 24 h urine catecholamine levels are easier to detect than intermittently raised plasma levels [6].

By combining urinary free noradrenaline measurement with plasma catecholamine suppression testing, high sensitivity (low false negatives) and high specificity (low false positives) are obtainable. Since levels of catecholamines in plasma and urine are substantially different, to date this has required two different LC systems. The capability for assay of both types of sample in one system is extremely attractive, and was the goal of this study.

Methods and Materials

Apparatus

Pump: LKB model 5120 (Pharmacia, UK) plus an additional pulse dampener (Waters Associates, Watford, UK). Injector: Gilson auto-sampler (Anachem, Luton, UK) with a 50-μl injection loop. Columns: (100 mm × 4.6 mm i.d.) 316 stainless steel (HETP, Macclesfield, UK). Detector: Waters M460 electrochemical detector (Waters Associates, Watford, UK) equipped with a glassy carbon working electrode. Integrator: HP 3390A integrator (Hewlett Packard, UK). All parts of the HPLC system were electrically grounded to a common point.

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Chemicals

Alumina type WA4, 3,4-dihydroxybenzylamine (DHBA), noradrenaline (NA), adrenaline (ADR) and dopamine (DA) were obtained from Sigma Company (Poole, UK). Potassium dihydrogen orthophosphate, trisodium citrate, phosphoric acid, EDTA, hydrochloric acid, TRIS and 1-octane sulphonic acid (OSA), all Analar grade, were obtained from BDH (Poole, UK), and methanol and ethyl acetate (HPLC grade) were obtained from Rathburn Chemicals (Walkerburn, UK). Plastic LP3 tubes were obtained from Luckhams (Burgess Hill, UK).

Chromatographic system

Analytical columns were packed with 3-μm ODS Hypersil (Shandon, Runcorn, UK) according to manufacturers instructions using a constant pressure Shandon Column Packer. The optimized mobile phase consisted of aqueous potassium dihydrogen orthophosphate (40 mM) trisodium citrate (40 mM) buffer, containing OSA (0.5 mM) and EDTA (4 mM, pH 6.4) methanol (90:10, v/v). Water was glass distilled and freshly deionized. The eluent was filtered and degassed prior to use and the flow rate was 1.2 ml min^{-1}. The working electrode was operated at potentials ranging from +0.65 to +0.90 V versus a Ag/AgCl reference electrode. The exact potential was determined by construction of a response versus voltage voltammogram after each cleaning of the working electrode and also after prolonged use. The detector sensitivity range was set at 0.5 nA V^{-1}.

Sample extraction

Plasma and urine samples required different pre-extraction handling procedures; however the subsequent extraction procedure used the same reagents and the same chemical basis.

Plasma extraction. Blood samples were drawn from subjects who were lying down and cannulated for 30 min. The plasma was flash frozen after separation from heparinized whole blood and stored at −70°C until analysis. Plasma samples were then thawed and centrifuged to remove insoluble matter. The extraction method for plasma catecholamines was as previously described [7].

Urinary extraction. The plasma extraction procedure was modified for use with urine samples. Urine was collected over 24 h into a bottle containing 20 ml 6 M HCl. After noting the total volume, a 100-ml aliquot was stored at −20°C until analysis. A 5-ml aliquot of urine was removed to a 20-ml stoppered glass tube. To this was added the internal standard (DHBA 150 pmol ml^{-1} urine) and 10 ml ethyl acetate. The mixture was shaken for 20 min, centrifuged to fully separate the organic and aqueous layers and the organic layer aspirated. One 500-μl and one 100-μl aliquot of aqueous phase were transferred to two LP3 tubes containing 10 mg alumina and sufficient Tris buffer (1 M; pH 8.6) to raise the pH to 8.6 (approx. 2 ml of Tris were enough). The samples were then shaken, washed and the supernatant aspirated as for plasma. 200 μl of phosphoric acid (0.5 M) were added to desorb the catecholamines from the alumina. The tubes were vortexed for 20 s and then centrifuged at 3000 rpm for 5 min. A 70-μl aliquot of supernatant was removed to a sample vial which was placed in the autosampler and 50 μl were injected.

Results and Discussion

Linearity

The operating voltage and detector range were optimized to give approximately full scale response for a 1-pmol injection of catecholamines. The linear range was determined by constructing a range of dilutions (n = 15) by weight from a top standard containing 200 pmol of each catecholamine per 50 μl. Table 1 shows the linear regression analyses of the results obtained. The response was linear over the range 0–200 pmol injected.

The limits of detection of a clinically useful catecholamine assay should be such that detection of the very low levels of plasma catecholamines is possible and this is adequately safeguarded by extraction of 2 ml of

Table 1
Linearity of catecholamine assays

Catecholamine	Slope	Correlation coefficient
Noradrenaline	22819	0.9939
Adrenaline	21887	0.9934
Dopamine	19838	0.9953
DHBA	12299	0.9942

Data from linear regression analysis of electrochemical detector response (integrator peak height) to catecholamines' concentration range 0–200 pmol injected. Intercepts in all cases were set at 0.

plasma. Urinary catecholamine levels vary over 3 orders of magnitude. A recent survey of normal ranges for urinary catecholamines reported mean values ranging from 80 to 200 nmol l^{-1} NA, 25–50 nmol l^{-1} ADR and 1500–2000 nmol l^{-1} DA [1].

Recovery

In order to use the existing plasma assay for urinary catecholamine analysis, the urinary concentrations of noradrenaline and dopamine require dilution so that their final injected concentrations fall within the linear range of the electrochemical detector. The final concentration injected onto the column (pmol/50 μl) is a function of the initial concentration, volume extracted, fraction of back-extraction acid volume injected, and the recovery. Absolute recovery was determined by replicate extractions of blank urine, which was spiked such that it contained 100 pmol ml^{-1} of each catecholamine. A 10-ml aliquot of this urine was extracted with 20 ml ethyl acetate, the phases allowed to separate and the organic phase aspirated; six 1-ml aliquots were then extracted and analysed as described above. The mean recoveries for noradrenaline, adrenaline, dopamine and DHBA were 52.8, 49.2, 52.2 and 56.1%, respectively.

From these recovery data the range of concentrations in urine and plasma which may be reliably reported as falling in the linear range of the assay was calculated. Since the limits of linearity of the assay are 0 to 200 pmol/injection, it was possible to reliably report values in the range 0.05–375 pmol ml^{-1} for a 2-ml plasma sample and for urine, 0.40–3000 pmol ml^{-1} for a 0.5-ml aliquot and 2.0–15,000 pmol ml^{-1} for a 0.1-ml aliquot. These ranges adequately covered the reported normal values and were sufficient to reliably report supranormal levels of catecholamines.

Reproducibility

The coefficient of variation was determined (n = 8) as NA 1.5%, ADR 1.7% and DA 0.8% for 25 pmol standards, NA 3.7%, ADR 5.1% and DA 4.8% for 0.1 ml urine extractions and NA 6.6% and ADR 7.5% for 2 ml normal plasma, in which DA was undetectable.

Extraction procedure

Using solely alumina extraction as for plasma, urine gave a very complex chromatogram with many peaks, some of which interfered with the catecholamine peaks (Fig. 1a). Washing with ethyl acetate prior to alumina extraction and careful washing of alumina before back extraction into acid produced a cleaner chromatogram (Fig. 1b).

Extraction of a 0.5-ml aliquot gave a lower limit of detection but 0.1-ml aliquot extraction produced a clearer chromatogram, possibly

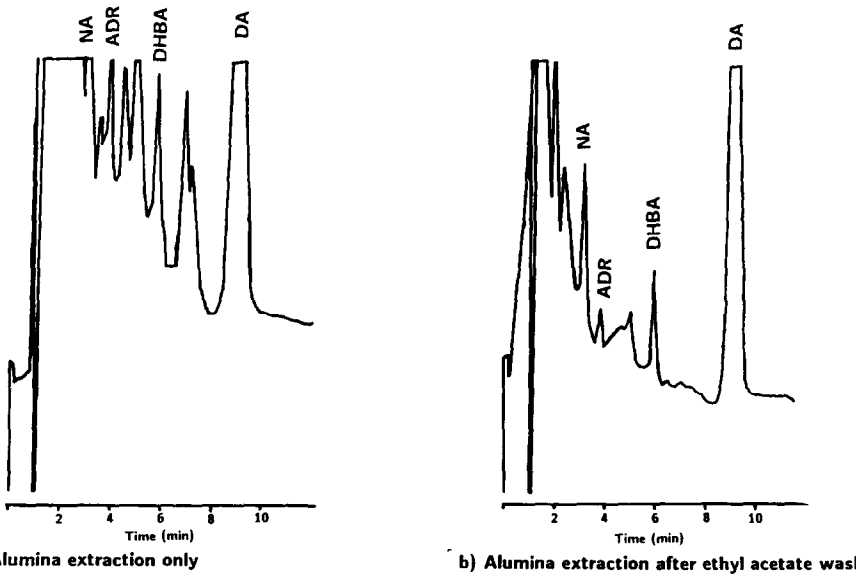

Figure 1
Chromatograms of 0.1-ml aliquots of urine extracted as described in Methods (a) without and (b) with ethyl acetate prewash. Detector operated at +0.70 V. Other conditions as per Methods section. (NA, noradrenaline; ADR, adrenaline; DHBA, dyhydroxybenzylamine; DA, dopamine).

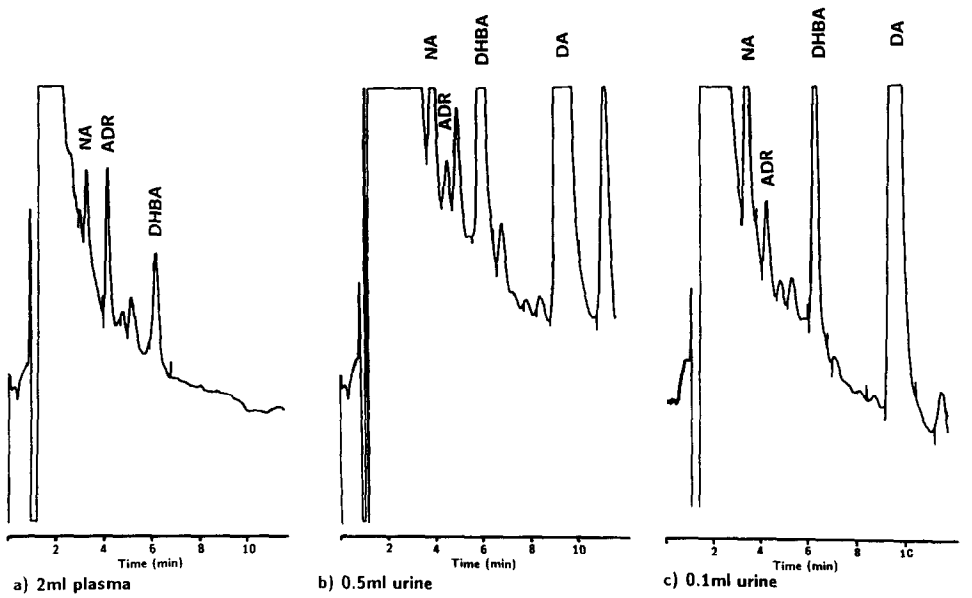

Figure 2
Sequential analysis of plasma and urine samples extracted as described in Methods without operator intervention. Conditions as in Fig. 1. Peaks are as identified in Fig. 1.

due to its greatly reduced carry-over of other urine constituents and increased dilution factor during washes and desorption (Figs 2b and 2c). Chromatograms of plasma and urine extractions were run consecutively using automated injection without operator attendance or interference (Fig. 2).

Applications to diagnosis of phaeochromocytoma

Ranges for urinary and plasma catecholamines were calculated from groups of normal subjects and hospital inpatients. From these data, warning limits were determined for plasma and urinary catecholamines (Table 2). Patients with levels exceeding these limits were then further investigated for phaeochromocytoma using suppression testing or imaging.

Table 2
Warning limits for plasma and urinary catecholamines

	Plasma (nmol l^{-1}) (n = 353)	Urine (nmol/24 h) (n = 66)
Noradrenaline	2.85 (0.09–6.09)	265 (45–466)
Adrenaline	1.00 (0.03–6.03)	62 (5–97)
Dopamine	Undetectable	2522 (364–4200)

Warning limits calculated as mean +2 SD for plasma and mean +1 SD for urine. Observed ranges in subjects without phaeochromocytoma in brackets.

Conclusions

The use of a single LC–EC system and uniform chromatographic conditions permitted plasma and urine samples to be extracted simultaneously and analysed sequentially in any order with minimal carryover. All samples were prepared by alumina extraction. High concentrations of catecholamines in urine could be compensated for by decreasing the volume extracted, and increasing the volume of back-extraction acid. Washing urine samples with ethyl acetate prior to alumina extraction reduced contaminant peaks. Use of 0.1 ml of washed urine gave adequate sensitivity for detection of low levels of noradrenaline and adrenaline as well as the high levels of dopamine, and kept all concentrations injected within the linear range of detection. Extraction of such small volumes of washed urine resulted in clean chromatograms. The detector response to NA, ADR and DA was linear over the range 0–200 pmol injected, corresponding to 0.05–400 pmol ml^{-1} for a 2-ml extraction of plasma, and 2–15,000 pmol ml^{-1} for a 0.1-ml extraction of ethyl acetate washed urine. This volume of urine was routinely used in the assay but 0.5 ml was used for adrenaline determinations in research studies.

The single, bi-functional, LC system described here is economically attractive and

provides greater sensitivity and versatility in investigation of phaeochromocytoma for the clinical chemistry laboratory. By incorporating an acid or enzymic hydrolysis step prior to organic extraction, total urinary catecholamines can also be determined.

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