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Short communication

Interferences by anti-TB drugs in a validated HPLC assay for urinary catecholamines and their successful removal

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ABSTRACT

A validated high performance liquid chromatographic assay for urinary catecholamines is presented. After addition of 3,4-dihydroxybenzylamine as internal standard (IS) to urine, norepinephrine (NE), epinephrine (E), dopamine (DA) are extracted by ion exchange chromatography and eluted with boric acid. After paired ion separation, quantitation is by electrochemical (coulometric) detection after correction of internal standard recovery. Novel interferences by anti-TB drugs on norepinephrine assay are discussed. A simple method for their removal using alumina is presented.

1. Introduction

Catecholamines epinephrine (E), dopamine (DA) and norepinephrine (NE) [1] determination in body fluids is used to help identify neuroendocrine disorders and other physiologic and pathologic conditions, such as pheochromocytoma [2].

Several methods described for assay of urine catecholamines [3,4] include reversed-phase liquid chromatography (LC) with electrochemical detection [5] or fluorescent detection [6]. Such analyses suffer from interferences from drugs and dietary constituents.

Traditional biochemical tests for detection of Pheochromocytoma rely on assay of 24 h urinary excretion of free catecholamines [7].

Aside from tandem mass spectrometry [8], there are currently no drug interference free-HPLC methods available for the rapid analysis of catecholamines. Although their LC–MS–MS analysis [8] of catecholamines was shown to be free of interference from many drug and drug metabolites, anti-TB drugs, like Rifampicin (RMP), were not tested for potential interference.

This paper reports a validated HPLC assay for quantitation of urinary free catecholamines using electrochemical detection and interference by anti-TB drug. To date no such reports on the latter interference has been thus far reported. A simple extraction procedure for removal of the interferent drug and (or) metabolite is described.

Ethics approval for the study (reference number: BE087/08) was given by the Biomedical Research Ethics Committee, University of KwaZulu-Natal.

2. Experimental

2.1. Chemicals

[−]-Epinephrine (+)-bitartrate salt, [−]-norepinephrine bitartrate salt, dopamine hydrochloride, 3,4-di-hydroxybenzylamine hydrobromide (internal standard), octanesulfonic acid sodium salt and aluminium oxide (powder, acidic Brockmann I activated standard grade, ~150 mesh) were obtained from Sigma (St. Louis, USA). Bio-Rex R 70 Resin (100–200 mesh sodium form) and Bio-Rad Lyphochek Quantitative Urine Control (Level 1 and Level 2) catecholamine quality controls were obtained from Rio-Rad Laboratories (Hercules, CA). External quality control material was obtained from the RCPAA EQA Program, Australia. All solvents and chemicals were of HPLC grade. Rifampicin (RMP) tablet (200 mg active) was obtained from IALC Hospital Pharmacy.

2.2. Instrumentation and conditions

Water was obtained from a Milli-RO PLUS 30 purification system (Millipore, USA). HPLC analysis was performed on a PerkinElmer (PE) Series 200 HPLC System with a programmable ESA Coulochem

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II Multi-Electrode electrochemical Detector (equipped with Coulometric cells: Model 5011 Analytical Cell and Model 5021 Conditioning Cell) (Chelmsford, USA), with the α-hydrogen/palladium as the reference electrode. A TurboChrom Workstation (Version 6.2) software was used for data collection. Chromotographic separation was accomplished with a LichroCART LicroSpher 100 RP-18e (125 mm × 4 mm I.D., 5 um size) cartridge and a LichroCART LiChrospher 100 RP-18e (4 mm × 4 mm, 5 um) guard column (Merck).

2.3. Mobile phase

(1) Standard: The mobile phase consisted of 22.21 mM citric acid/0.38 mM EDTA/1.14 mM octanesulfonic acid sodium salt (in distilled water (18.2 MΩ) adjusted to pH 5 with 2 M sodium hydroxide)-methanol (900:100 v/v) at flow rate, 1.1 ml/min and column temperature 35 °C, with a run time of approximately 15 min. (2) Slow: A "slow" phase was prepared as above using 900 ml of buffer (pH 5) and 50 ml of methanol for analysis of urine samples from patients on anti-TB treatment, at flow rate of 1.1 ml/min, maintained at 35 °C with a run time of approximately 40 min.

2.4. Standard solutions

2.4.1. Catecholamines

External standard preparation: stock standard: separate stock standards were prepared to give final concentrations of 0.200 mg/ml norepinephrine, 0.120 mg/ml epinephrine, 0.200 mg/ml dihydroxybenzylamine and 0.800 mg/ml dopamine in 0.1 M HCl.

Working external Stock: The above solutions were combined and diluted with 0.01 M HCl to give final concentrations of 1 μg/ml norepinephrine, 0.6 μg/ml epinephrine, 1 μg/ml dihydroxybenzylamine and 4 μg/ml dopamine. Working external standard: was prepared daily in 2% boric acid by a 1/25 dilution of the Working external stock to give final concentrations of 40 ng/ml norepinephrine, 24 ng/ml epinephrine, 40 ng/ml dihydroxybenzylamine and 160 ng/ml dopamine. Internal standard: stock internal standard: A stock internal standard (IS) was prepared by dissolving ~15–20 mg of dihydroxybenzylamine hydrobromide in 0.1 M HCl to give a final concentration of 1.00 mg/ml dihydroxybenzylamine.

Diluted stock internal standard: the stock internal standard was diluted 1/10 in 0.1 M HCl to give a 0.1 mg/ml solution of dihydroxybenzylamine.

Working internal standard was prepared daily by a 1/100 dilution of diluted stock internal standard solution in water.

2.4.2. Anti-TB drugs: Rifampicin

A Stock standard solution of 1 mg/ml was prepared in methanol. Subsequent solutions were prepared, after drying of the stock with nitrogen, in HCl and boric acid, for spiking urine samples and for HPLC retention time analyses.

2.5. Urine samples

Twenty four-hour samples from subjects, collected in amber bottles containing 100 ml 2 M HCl, were stored at 4 °C for 1–2 d or at −20 °C for longer periods.

2.6. Sample preparation

Procedure 1: Bio-Rex 70 columns, packed with 0.6 g resin (headspace of 15 ml) were conditioned with 30 ml 1 M HCl, 5 ml water, 30 ml 0.5 M NaOH and 30 ml phosphate buffer pH 6.5 (0.20 M Na_2HPO_4, containing 0.025% sodium azide, adjusted to pH 6.5 with concentrated phosphoric acid) prior to storage at 4 °C. Before use, columns were warmed to room temperature and treated with 10 ml phosphate buffer pH 6.5 and 15 ml water. A mixture of 3.0 ml urine (sample), 300 μl working internal standard solution and 10.0 ml stabilising solution (0.1% EDTA in water) was adjusted to pH 5–6.5 with 2 M NaOH. The entire volume was applied to the Bio-Rex column. After drainage, the column was washed with 2 × 15 ml distilled water. The catecholamines were eluted with 6 ml of 2% boric acid. Sample extract (1 ml) was filtered through a 2 ml syringe filter (0.45 um). Ten microlitres of extract (or external standard) were injected into the HPLC system for analysis.

Procedure 2: Boric acid extract (4 ml), from Procedure 1 was added to a kimax tube containing alumina (100 mg), tris buffer pH 8.6 (500 μl), 4% thioglycolic acid (100 μl) [9]. The stoppered tube was mixed on a shaker for 10 min followed by centrifugation at 4000 rpm for 3 min. The liquid phase was discarded. Water (2 ml) was added to the tube which was then shaken for 2 min. After centrifugation at 4000 rpm for 3 min, the water was discarded. The water wash was repeated. After removal of the water, 1 M acetic acid (1 ml) was added to the tube, followed by subsequent shaking for 10 min. After centrifugation, the acid layer was filtered and assayed by HPLC.

3. Results and discussion

3.1. Method validation criteria

3.1.1. Analytical potential

The following conditions gave optimum signal: noise response: guard cell: +350 mV, DC channel 1: E: 0 mV, *R*: 200 nA, Filter: 5 s, Ch1 output: 1 V, offset: 5%; DC channel 2: E: +450 mV, *R*: 200 nA, filter: 5 s, Ch2 output: 1 V, offset: 5%. A potential of +450 mV was found to be optimum for analysis.

3.1.2. Choice of peak area versus peak height

Peak height integration was found to be superior to peak area integration.

3.1.3. Specificity

The resulting chromatograms were essentially free from endogenous interference. The retention times averaged 3.923 ± 0.091, 5.173 ± 0.140, 7.133 ± 0.214 and 11.350 ± 0.385 min for NE, E, IS and DA respectively (CV = 2.33, 2.70, 3.00, 3.39 for $n = 20$, intra-day variation). Fig. 1 shows the chromatogram of a standard solution of amines. The assay was shown to be free of interference from the anti-hypertensive drug Aldomet.

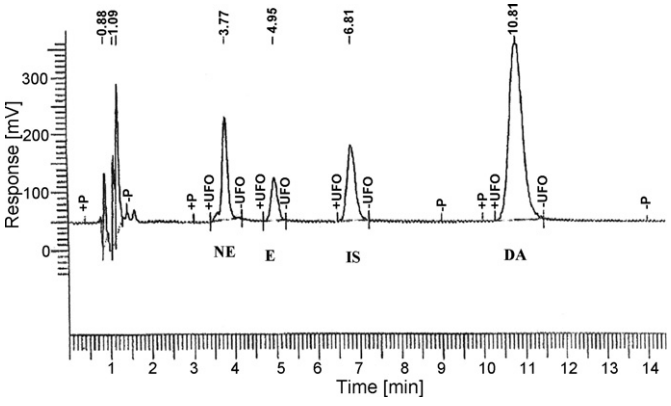

Fig. 1. Chromatogram of external standard: norepinephrine (RT 3.77 min), epinephrine (RT 4.96 min), IS (RT 6.81 min), dopamine (RT 10.81 min).

3.1.4. Linear range

Standard solutions prepared in dilute acid were used. The data were fitted to a line by the equation $y = ax + b$ where y is the peak height, b, the intercept and a the slope. Regression analysis showed good linearity. The correlation coefficients averaged 0.999477 (SD = 0.000458, CV% = 0.05) for all three catecholamines for a concentration range of 1–150, 3–50 and 3–625 μg/L for norepinephrine, epinephrine and dopamine, respectively.

3.1.5. Limit of detection (LOD) and limit of quantitation (LOQ)

Using standards, at a minimum signal:noise ratio of 3.0:1, the limit of detection was found to be 1 μg/L (6 nmol/L), 3 μg/L (16 nmol/L) and 3 μg/L (20 nmol/L) for norepinephrine, epinephrine and dopamine, respectively. The LOQ was evaluated as the minimum concentration detectable at a signal/noise ratio of ≥10:1 to be approximately 9 μg/L (53 nmol/L), 11 μg/L (60 nmol/L) and 16 μg/L (105 nmol/L) for norepinephrine, epinephrine and dopamine, respectively.

Although conventional coulometric detectors give generally picomolar detection limits, sensitivity of electrochemical detectors is inversely related to experimentally observed oxidation (or reduction) potentials [10]. Amperometric detectors have the advantage of being more sensitive and less complex than the coulometric detectors, used in this study. Earlier work using coulometric detectors reported detection limits of 6600, 5500 and 7200 [9] and 5900, 54,600 pmol/L [11] (DA not reported) for NE, E, and DA, respectively. For chromatographic detection, sample preparation and injection are normally primary sources of error and relative measurements (internal or external standards) predominate. Thus, the lower than expected detection limits obtained may be due to the latter.

3.1.6. Stability

All stock standards and working standards were stable at 4 °C for 3 months.

3.1.7. Accuracy

For Bio-Rad urine controls, due to the target values of the Level 2 quality control material exceeding the detector linear range, a 1/3 dilution in water of control material was used for assay. The within-day precision for $n = 9$ aliquots for Level 1 and Level 2 is listed in Table 1. Overall accuracy for all three amines was 94%. For external Australian quality control (AQC) samples, the correlation statistics observed were as follows: NE: $r^2 = 0.994488$, $a = 1.035086$, $b = -22.62693$; E: $r^2 = 0.983185$, $a = 1.006967$, $b = -44.58037$; DA: $r^2 = 0.962675$, $a = 1.024471$, $b = -0.04769$. Regression analysis showed good linearity. The correlation coefficients averaged 0.980116 (SD = 0.016127, CV% = 1.65) for all three catecholamines. The corresponding t-test values were 0.2222, 0.8956, and 0.0029 for NE, E and DA, respectively. All t-values were less than 2.0244, at a P-value of 0.050 showing that observed assay values were not significantly different from the EQA median values. The mean extraction recoveries were 93%, 97% and 88% (overall 92%) for NE, E and DA, respectively, using Procedure 1. The corresponding recovery for the IS averaged 88%, 92% and 89% (overall 90%). Overall accuracy was 93% for all three amines.

3.1.8. Precision

For Bio-Rad controls, the within-day (inter-day) imprecision observed were 2.38%, 2.81% and 1.45% for Level 1 (Lot 62191) and were 1.76%, 2.22% and 1.60% for Level 2 (Lot 62192) for norepinephrine, epinephrine and dopamine, respectively for 10 samples ($n = 10$). The corresponding day-to-day (intra) imprecision were 5.13%, 6.25% and 5.33% for Level 1 (Lot 62191) and 3.85%, 7.41%, and 5.51% for Level 2 (Lot 62192), respectively over 1 month period ($n = 20$). For real samples, the within-day mean CV% was 4.64% for three different urine samples ($n = 5$) for the range: 15–288, 0–5 and 59–451 μg/24 h for NE, E and DA, respectively.

3.2. Application

3.2.1. Pheochromocytoma

A urine sample from a patient with a left adrenal pheochromocytoma, on no TB medication, had typical assay values of 2 535 nmol/d NE, 486 nmol/d E and 2.20 umol/d DA.

3.2.2. Interferences by TB drugs

Rifafour contains Rifampicin, Isoniazide, Ethambutol and Pyrazinamide. Rifinah contains Rifampicin and Isoniazide. Earlier work (self) involved spiking urine with Rifafour, Rifinah, Isoniazide, Ethambutol, Pyrazinamide to exclude evidence of interference by the other components: Isoniazide, Ethambutol, Pyrazinamide. Only Rifafour and Rifinah, both containing active Rifampicin, showed "interference" on the spiked urine chromatograms at the elution time of NE. The chromatogram of an external standard solution of amines (not shown) showed NE eluting at 4.13 min with the standard mobile phase. The corresponding chromatogram of a patient urine sample (not shown), the patient being treated with Rifafour, showed NE and "interferent" eluting at 4.09 min. The observed assay values were 209, less than 4 and 120 μg/24 h for NE, E, and DA, respectively. Using the "slow" mobile phase, the observed assay values were 21 (NE elutes at 6.21 with "slow" phase), less than 4 and 106 μg/24 h for NE, E and DA, respectively.

Table 1
Assay values (μg/24 h) of urine from patient on anti-TB drug regimen before and after alumina extraction

Urine specimen number	Standard phase			Slow phase			Al_2O_3/standard phase		
	NE	E	DA	NE	E	DA	NE	E	DA
513	139	8	193	*28*	nd	360	*31*	nd	429
	201	6	363				30	5	343
	215	6	358				31	6	345
	248	6	483				30	6	286
514	270	9	411	*30*	nd	402	*32*	10	439
	475	10	392				33	10	293
	452	8	386				31	10	451
	667	7	613				31	10	301
516	218	7	360	*14*	5	358	*27*	5	256
	200	8	344				27	7	352
	190	8	344				27	7	279
	192	8	454				27	7	283

nd: not detected.

A normotensive patient urine sample was spiked with Rifampicin at 200 μg/ml, and stored at 4 °C. A blank, unspiked urine sample (same patient) was retained at 4 °C to determine interference of the TB drug. Both samples were then processed by Procedure 1. A further additional urine sample, spiked with Rifampicin, was extracted with chloroform [9] prior to ion exchange resin purification, in an attempt to remove the drug. The latter procedure was unsuccessful. The chromatogram (not shown) of the standard solution of Rifampicin in acid shows a peak eluting at 4.01 min, equivalent to the retention time of norepinephrine with the standard phase. Initial assay values were 25, 5 and 236 μg/24 h for NE, E and DA on the unspiked urine sample. Final assay values were 52, 5 and 243 μg/24 h for NE, E, DA on the spiked, processed urine sample, showing elevation of the norepinephrine assay value from 25 to 52 μg/24 h.

Cation-exchange chromatography is suitable as a one-step sample clean-up for analysis of free and total catecholamines [9]. Our method is based on the use of Bio-Rex 70, a cation-exchange resin [12]. The latter report [12] investigated the chromatographic behaviour of catecholamine metabolites and analogues on the cation exchange column. Acid metabolites were not retained by the resin and thus did not interfere with the assay [12]. Similar results were obtained for the alcoholic metabolites and the amino acids. Although all amines tested were retained by the resin, only those compounds containing a vicinal hydroxyl group were retained by the resin and eluted with boric acid: alphamethylnorepinephrine, *N*-methyldopamine, salsinol, alphamethyldopamine, isoproterenol and tetrahydropapaveroline [12].

Isoniazide and Pyrazinamide lack hydroxyl group functionalities. The chromatogram (not shown) of the urine from a patient on Rifampicin (and Isoniazid, Pyrazinamide, Ethambutol), analysed by the "slow" phase showed a peak for norepinephrine, eluting at 6.21 min, whilst a major component elutes at retention time 6.54 min. The external standard chromatogram indicates that NE elutes at 6.22 min. The peak eluting at 6.54 min in the patient chromatogram is an interferent.

A modified procedure, initially reported by Kissinger and Riggin [9], using alumina [13], was found to be effective in removal of the drug and (or) metabolite from the boric acid extract. Three urine 24 h collections from another patient, on Rifampicin (and Isoniazide, Ethambutol, Pyrazinamide), were submitted to our laboratory for assay of catecholamines, for assistance in diagnosis of pheochromocytoma pheochromocytoma. The samples were processed using Procedure 1 and Procedure 2. The external standard chromatogram (not shown) shows peaks at retention time 3.31 min (NE), 4.26 min (E), 5.78 min (IS), 9.07 min (DA). Fig. 2 shows one

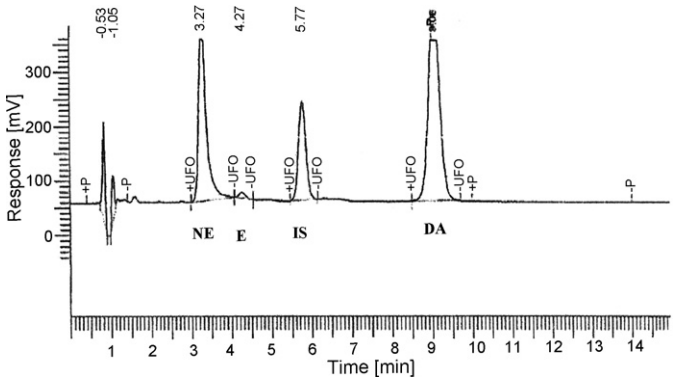

Fig. 2. Chromatogram of a patient on anti-TB regimen: "norepinephrine"/drug interferent (RT 3.27 min), epinephrine (RT 4.27 min), IS (RT 5.77 min) and dopamine (RT 9.06 min).

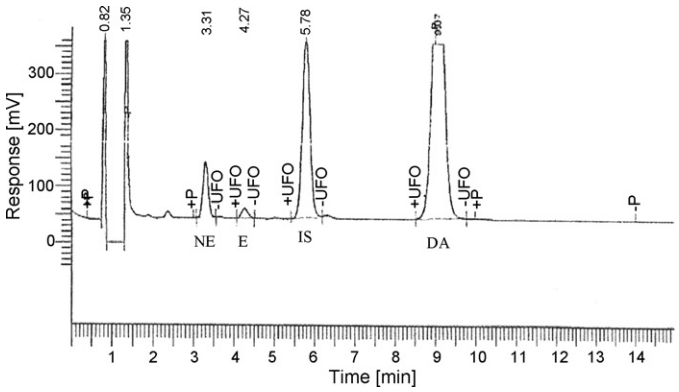

Fig. 3. Chromatogram of a patient on anti-TB regimen, after alumina extraction–standard phase: norepinephrine (RT 3.31 min), epinephrine (RT 4.27 min), IS (RT 5.79 min) and dopamine (RT 9.07 min).

of the urine sample chromatogram after normal extraction (Procedure 1): peaks at retention time 3.27 (NE, "drug interferent), 4.27 min (E), 5.77 min (IS), 9.06 min (DA). Initial assay values were: NE = 170 μg/24 h, E = 8 μg/24 h, DA = 351 μg/24 h. Fig. 3. shows the urine sample chromatogram after alumina extraction–Standard phase (Procedure 2): peaks at retention time 3.31 min (NE), 4.27 min (E), 5.79 min (IS), 9.07 min (DA). Final assay values were now: NE = 27 μg/24 h, E = 7 μg/24 h, DA = 289 μg/24 h. Obvious is the peak at retention time 3.31 min, which now, after removal of the "drug interferent", matches the time at 3.31 min on the external standard, corresponding to NE.

Other results on different urine collections assayed for this patient are listed in Table 1. Mean values for NE using the slow phase and alumina extraction–standard phase were compared (31 versus 28, 32 versus 29, 27 versus 19). Values for the other two amines, E and DA, were also compared using the standard phase and alumina extraction–standard phase procedure. Paired *t*-test (statistical data available) showed that the data are not significantly different. The *p*-values are all >0.05. Mean recoveries for the internal standard averaged 81% for urine samples, containing TB drug, and quality control material.

For Rifampicin, with a dose of 900 mg, about 25% of Rifampicin may be excreted in urine [14]. Principal pathways of metabolism are desacetylation and hydrolysis. Desacetylation at C-25 position results in a compound with increased capacity for biliary excretion. Depending on dose, one third to one eight may be excreted in bile either as unchanged desacetylrifampicin (DRMP) or as unchanged Rifampicin. The latter is reabsorbed whereas the 25-*O*-desacetyl derivative is poorly absorbed. Acid-catalysed hydrolysis of Rifampicin has been reported [15], forming 3-Formylrifamycin.

In the HPLC determination of Rifampicin and DRMP, Hemanth Kumar et al. [16] reported retention times of approximately 2.9 and 4.8 min for DRMP and RMP, respectively under similar chromatographic conditions as us. Similarly, Panchagnula et al. [17] reported retention times of approximately 4.0 and 6.0 min for DRMP and RMP, respectively.

Our results indicate that Rifampicin (and (or) metabolites) is retained by the ion exchange column and subsequently eluted with boric acid to produce the observed interference at the elution time of norepinephrine in the chromatogram of the patient sample.

Initial assay values for quality control using standard cleanup (Procedure 1) were 45 and 175, 16 and 89, 76 and 426 μg/L for NE, E, DA, for Level 1 and Level 2. After subsequent extraction of the boric acid extract using alumina (Procedure 2), the values were 42, 13, 57 and 175, 75, 342 μg/L assay values for NE, E, DA for Level 1 and Level 2 respectively, indicating reduced recovery and accu-

racy. Corresponding laboratory computed ranges were 43–47 and 175–199, 14–18 and 870–99, 69–85 and 394–482 (μg/L) for NE, E, DA for Level 1 and Level 2, respectively. Thus, loss of catecholamine occurs during the alumina extraction step.

4. Conclusion

Extraction of catecholamines from urine using alumina is not popular. Early reports [9,11] using a two-step clean-up procedure (cation exchange columns, followed by alumina) indicated poor recoveries (51% and 60%), presumably due to the basic pH involved (above 8.5) at which catecholamines are unstable.

Future work should investigate the potential use of alumina alone to extract both catecholamines and TB drug from urine in one-step. Based on initial results obtained, the latter does not appear promising due to reduced recovery observed.

Although the increased sensitivity, and specificity, benefits gained by use of HPLC–MS methods for catecholamine assay are appealing, the cost implications of such equipment in a routine clinical service setting may be a problem.

An accurate, precise validated HPLC method for simultaneous determination of urinary free catecholamines for diagnosis of pheochromocytoma was developed. Alumina can successfully remove interference by TB drugs, like Refampicin, on assay determination of norepinephrine, from urine of such patients, enabling an accurate assay value for NE, in a much shorter time period.

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