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ROUTINE DETERMINATION OF URINARY FREE CATECHOLAMINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A simple and reliable high-performance liquid chromatographic method is described for the routine determination of the free catecholamines (norepinephrine, epinephrine and dopamine) in urine. The catecholamines are isolated from urine samples using small affinity chromatography columns pre-packed with immobilised *m*-aminophenylboronic acid, separated by ion-pair reversed-phase liquid chromatography and quantified by electrochemical detection. Total analysis, including sample preparation time, is achieved in less then 30 min with analytical recoveries of 92–96% for all three catecholamines. Long-term stability and reproducibility of the liquid chromatographic system is attained by selection of optimised conditions for chromatographic separation with a formate mobile phase and produces detection limits of 1.4, 1.8 and 2.2 nmol/l for norepinephrine, epinephrine and dopamine, respectively, in urine samples and day-to-day coefficients of variation of less than 6%. Furthermore, the affinity isolation gels can be reused a minimum of ten times providing a rapid and cost-effective means of sample preparation.

INTRODUCTION

Recent reports [1,2] have indicated the advantages of catecholamine analysis in the detection of neoplasms of neuroectodermal origin, such as pheochromocytoma. Moreover, the estimation of dopamine (DA), norepinephrine (NE) and epinephrine (E) levels in essential hypertension and neurological disorders is well established. Although many high-performance liquid chromatographic (HPLC) procedures have been described for the chromatographic separation of catecholamines [3–5], a frequent disadvantage of many procedures is the occurrence of interfering peaks on the chromatogram due to the sample pre-treatment method. An extensive sample clean-up is invariably required to effectively isolate the catecholamines with sufficient sensitivity and minimise the simultaneous iso-

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lation of interfering compounds. However, many sample preparation procedures fail to achieve these objectives. Sample pre-treatment can involve isolation on activated alumina [6,7] or the use of gravity-fed columns packed with cation-exchange resins [8,9], boronate gels [10] or Sephadex [11].

In selecting a procedure suitable for the routine and long-term investigation of urine catecholamines, several disadvantages of current methodology were apparent: limited isolation specificity, tedious sample preparation, poor resolution of compounds of interest and relatively long chromatogram run times. To resolve these problems, a procedure using a rapid and simple sample preparation step in conjunction with optimised HPLC separation has been developed to provide a reliable, reproducible and sensitive method suitable for the routine analysis of unconjugated catecholamines in urine.

EXPERIMENTAL

Chemicals

NE, E, DA, Tris base and the internal standard, 3,4-dihydroxybenzylamine (DHBA) were purchased from Sigma London (Poole, U.K.), while sodium 1-octane sulphonic acid (OSA) was obtained from Fisons Scientific (Loughborough, U.K.) and HPLC-grade methanol from Rathburn (Walkerburn, U.K.). All other reagents were analytical grade supplied by BDH (Poole, U.K.) with the exception of chemicals used for interference studies (Sigma).

α-Methylnorepinephrine and α-methyldopamine were a gift from Sterling-Winthrop (Guildford, U.K.). The pre-packed affinity chromatography columns for sample preparation, containing 0.5 ml of immobilised boronic acid (Glycotest 25), were purchased from Pierce (Cambridge, U.K.). All water was deionised, glass-distilled.

Reference solutions

Individual stock standards of NE, E and DA containing 20 mg/l (free base) were prepared in 0.1 *M* acetic acid every two months and stored at 4 °C. Combined working standard solutions were prepared for each analysis batch by dilution with deionised, distilled water. The internal standard, DHBA (20 mg/l) was prepared in 0.1 *M* acetic acid and stored at 4 °C. The DHBA stock solution was diluted weekly 100-fold in deionised, distilled water for use as an internal standard.

Apparatus

The liquid chromatographic system was composed of a Waters Assoc. Model 6000A pump with a Negretti injection valve fitted with a 50-μl sample loop (Waters Assoc., Harrow, U.K.). Chromatography was performed on a stainless-steel column, 150 mm $\times$ 4.5 mm, packed with 5-μm Hypersil ODS, and a guard column, 30 mm $\times$ 4.5 mm containing 10-μm octyldecylsilane bonded packing (Jones Chromatography, Mid Glamorgan, U.K.). Catecholamines were detected using a BAS LC-4B amperometric detector with a TL-5 thin-layer glassy carbon electrode set at an electrode potential of +0.65 V vs. an Ag/AgCl reference electrode (Bio-Analytical Systems, West Lafayette, IN, U.S.A.). Peak areas and quantification

were estimated with a Spectra-Physics SP4270 computing integrator (Spectra-Physics, St. Albans, U.K.).

Mobile phase

The chromatographic mobile phase was a methanol–buffer mixture (5:95, v/v), pH 4.0, with buffer composition of 50 m*M* sodium formate, 0.15% formic acid (99%), 0.1% triethylamine, 0.25 m*M* OSA and 0.2 m*M* disodium EDTA. The mobile phase was filtered through a 0.22-μm Type HA filter (Millipore, Bedford, MA, U.S.A.) on a daily basis for filtration and degassing. A minimal buffer flow-rate of 0.3 ml/min was maintained through the system at all times to minimise fouling of the electrode and maintain reproducible separation. Recycled, the mobile phase was used continuously for at least one week.

Sample preparation and chromatography

Urine samples for analysis were collected over a 24-h period in polyethylene containers, and 10–15 ml of 6 *M* hydrochloric acid were added as preservative. Samples were stored at −15°C until analysis. To 1 ml of urine sample and working standard solution were added 100 μl of DHBA internal standard followed by 200 μl of 2 *M* Tris buffer (pH 8.0, containing 0.2% disodium EDTA). Samples were verified to be within pH 7.5–8.0 after addition of 2 *M* Tris buffer and were adjusted if necessary with further addition of Tris buffer. To avoid potential losses of the catecholamines, samples were buffered individually before being immediately transferred to an affinity chromatography column (Glycotest 25) previously equilibrated at room temperature with 5 ml of 200 m*M* Tris pH 8.0. After adsorption of the sample, interfering compounds were removed by sequential washing of the affinity gels with 5 ml of 100 m*M* Tris (pH 8.0), 5 ml deionised, distilled water and 500 μl of 100 m*M* acetic acid, discarding all effluents. The catecholamines were subsequently eluted with 500 μl of 100 m*M* acetic acid, and 50-μl aliquots were injected into the chromatography system for analysis at a mobile phase flow-rate of 1.0 ml/min. Catecholamine concentrations were calculated using response ratios of standards processed through the extraction procedure and applied to peak-area ratios in the sample. The catecholamines were found to be stable in the acetic acid eluates for up to four days at 4°C. The affinity chromatography gels were regenerated immediately after use with 5 ml of 100 m*M* acetic acid followed by a further 2–3 ml of 50 m*M* acetic acid for storage in this solution at 4°C. When protected from excessive exposure to sunlight and regenerated in this way, the affinity gels can be reused at least ten times without any apparent loss in isolation capacity.

RESULTS

Typical chromatograms resulting from the injection of affinity gel eluates of an aqueous catecholamine standard and urine samples are shown in Fig. 1. The compounds are baseline-resolved in the analysis time of approximately 15 min with the selected HPLC conditions.

A formate buffer mobile phase was preferred to the more frequently used phos-

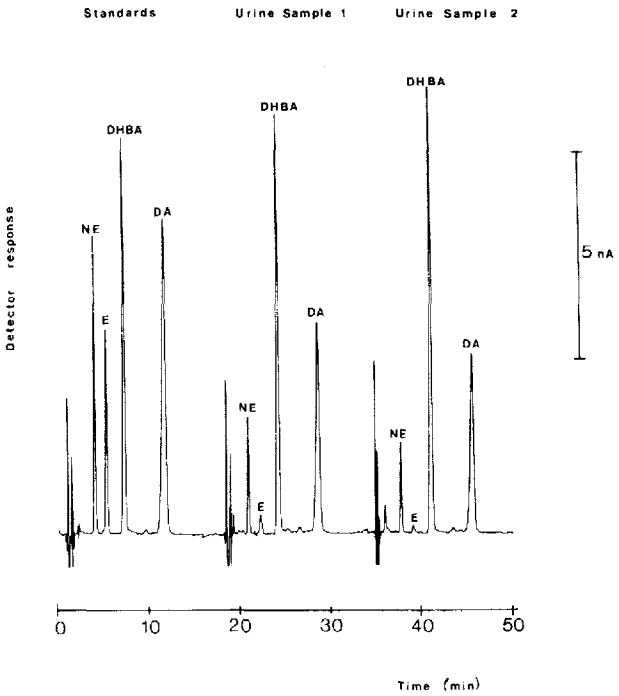

Fig. 1. Chromatograms of affinity gel eluates of a reference solution and two normal urine samples. Catecholamine concentrations in the urine samples correspond to the following levels: sample 1: 98, 12 and 462 nmol/l for NE, E and DA, respectively; sample 2: 73, 8 and 372 nmol/l for NE, E and DA, respectively.

phate, acetate or citrate buffers as it produced long-term stability of the HPLC packing in the presence of the ion-pairing reagent, OSA. Furthermore, it provided the necessary sensitivity and buffering capacity suitable for catecholamine analysis, and its volatility minimised potential damage to the chromatographic analysis that can occur with other buffer systems [12]. A systematic study of the influence of pH and OSA concentration on catecholamine retentions with the formate mobile phase is shown in Fig. 2, where the log of the capacity ratios of the catecholamines was plotted as a function of the pH of the mobile phase. A concentration of 0.25 m*M* OSA achieved baseline separation of the catecholamines in a minimum time period, and maximal detector sensitivity was found at a formate concentration of 0.05 *M*. The retention data were obtained at a methanol concentration of 5% as an increased concentration reduced the resolution between NE and E and a decreased concentration significantly affected peak symmetry. Hydrodynamic voltammograms of the three catecholamines and the internal standard (DHBA), obtained at different detector potentials with the formate mobile phase, demonstrated that an electrode potential of +0.65 V was optimal for detector sensitivity and low background signal. Identical voltammograms were also obtained for catecholamine reference solutions and affinity gel eluates of urine samples, thus verifying the purity of the isolation procedure.

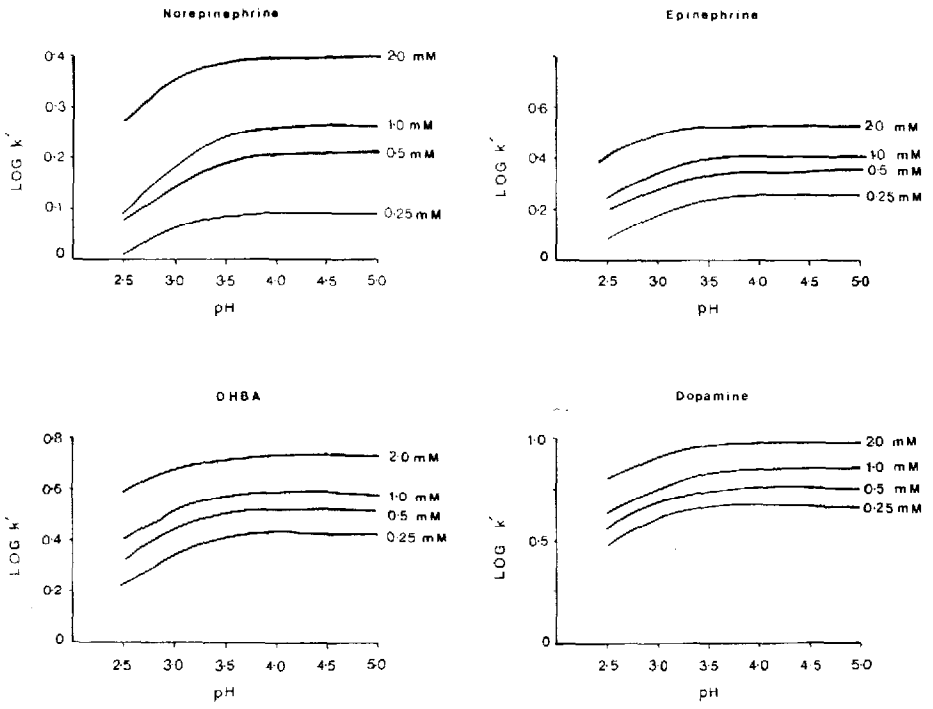

Fig. 2. Influence of pH and ion-pairing concentration (OSA) on the capacity ratios of NE, E, DA and DHBA. Stationary phase: Hypersil ODS; mobile phase: formate buffers (50 m*M*) with varying concentrations of OSA (0.25, 0.5, 1.0, 2.0 mmol/l) and 5% methanol.

Linearity and detection studies

The electrochemical response with the selected HPLC conditions was linear to at least 500 pmol for NE and E and 1000 pmol for DA. The linearity of the sample isolation procedure was assessed by the analysis of standards prepared in deionised, distilled water and in a catecholamine-free urine. The urine sample was rendered free of endogenous catecholamines by adjusting a suitable 24-h urine collection without preservative to pH 8.5 and adding sufficient alumina to ensure adsorption of the catecholamines. Peak-area ratios of NE, E and DA to internal standard were found to be linear throughout the range 0–10 000 nmol/l for NE and E and at least 0–25 000 nmol/l for DA. In addition, there was no difference in the calibration plots obtained when either aqueous or urine-based standards were plotted as ratios against concentration. Correlation coefficients (r) in the isolation and assay procedures were: NE, 0.999 and 0.997; E, 0.999 and 0.998; DA, 0.999 and 0.996 for aqueous and urine-based standards, respectively. The detection limits of the assay, assuming a signal-to-noise ratio of at least 4, were 1.4 nmol/l for NE, 1.8 nmol/l for E and 2.2 nmol/l for DA in native urine samples.

Optimisation of isolation procedure

Isolation of the catecholamines from urine and aqueous solutions was observed to be optimal at sample pH 7.5–8.0 and with the boronic acid gels at pH 8.0. Sample pH adjustment was readily achieved by the addition of high-molarity Tris

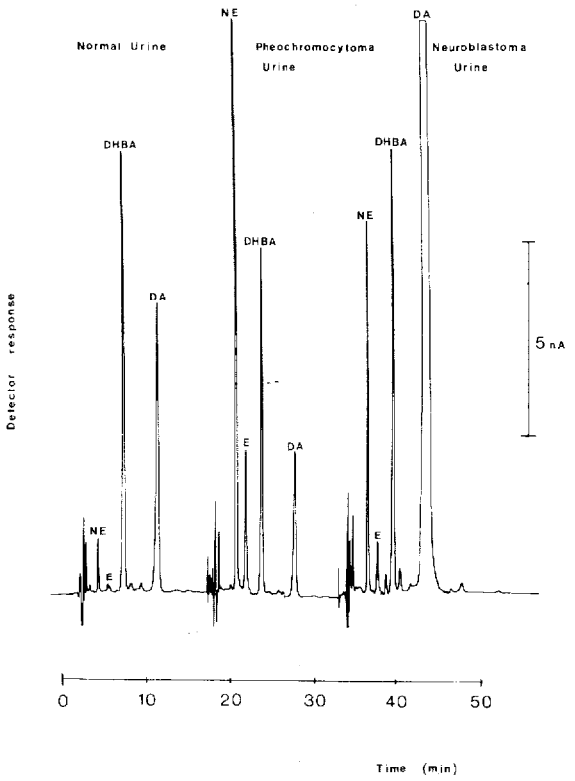

Fig. 3. Chromatograms obtained for a normal patient's urine sample and for urine samples from pheochromocytoma and neuroblastoma patients. Sample pre-treatment and HPLC mobile phase as described in the text.

buffer, as the choice of buffer species had no apparent influence on the absorptive properties of the boronic acid gels. The isolative capacity of the affinity gel columns was sufficient to determine both the levels encountered in normal samples and the elevated concentrations observed in neural crest tumour patients (Fig. 3).

Recovery and precision studies

Recoveries of the isolation step were determined by the addition of known amounts of catecholamines to patient urine samples ($n=15$) and to a catecholamine-free urine specimen. Mean recoveries obtained were at least 93% for all catecholamines, as shown in Table I.

The precision of the assay was assessed by the analysis of quality-control urine specimens and pooled urine samples stored in aliquots at $-70\,^{\circ}C$. The precision studies, performed over eighteen months with different batches of the pre-packed affinity gel columns and HPLC stationary phase, are summarised in Table II.

Interference studies

A combined investigation of the adsorption properties of the immobilised *m*-aminophenylboronic acid and the retention characteristics of the HPLC system

TABLE I

ANALYTICAL RECOVERIES OF CATECHOLAMINES ADDED TO URINE SAMPLES

Catecholamine	Recovery (mean ± S.D., $n=15$) (%)	Concentration range (nmol/l)
Norepinephrine	96.9 ± 3.2	59-1892
Epinephrine	95.1 ± 3.6	54-1092
Dopamine	93.8 ± 3.9	147-2940

TABLE II

PRECISION OF THE CATECHOLAMINE ASSAY

Values in parentheses are coefficients of variation (%).

Sample	n	Mean urine concentration (nmol/l)		
		Norepinephrine	Epinephrine	Dopamine
Level 1				
Within-assay	30	208.3 (3.5%)	79.8 (4.1%)	705 (3.4%)
Inter-assay	64	238.3 (5.3%)	76.8 (5.8%)	769.7 (3.8%)
Level 2				
Within-assay	30	882.5 (3.1%)	156.6 (3.6%)	1510 (2.9%)
Inter-assay	63	892.9 (4.1%)	165.5 (4.3%)	1515 (3.6%)
Level 3				
Within-assay	30	1128 (2.9%)	488.1 (3.0%)	2050.3 (2.3%)
Inter-assay	64	1114.7 (3.7%)	482.4 (4.2%)	2058.5 (3.6%)

was undertaken with catecholamine metabolites and analogues. The compounds were applied to the affinity gel columns, and the eluates were injected into the HPLC system for detection. The results are shown in Table III. With the selected conditions, compounds with a vicinal hydroxyl function were initially retained by the boronic acid while only amines with this configuration were still retained after the wash procedures.

Although α-methyldopa (Aldomet®), a frequently used antihypertensive drug, was not retained by the affinity gel columns, the synthetic catecholamine, isoproternol and α-methyldopa metabolites, such as α-methylnorepinephrine and α-methyldopamine, were retained. All these compounds are well resolved from the catecholamines with the selected HPLC conditions (Table III).

Correlation and population studies

Urine catecholamine results by the affinity gel procedure for a mixed group ($n=62$), composed of normal, hypertensive and neural crest tumour subjects, were compared with results obtained by an alternative HPLC procedure using cation-exchange isolation as a sample pre-treatment [8]. When alumina was used as a sample preparation step, quantification of the low levels of E frequently

TABLE III

CHROMATOGRAPHIC BEHAVIOUR AND RETENTION CHARACTERISTICS OF POTENTIAL INTERFERING COMPOUNDS

Compound	Retention on affinity gel	Elution with catecholamines	HPLC capacity factor (k')
4-Hydroxy-3-methoxymandelic acid (HMMA)	–	–	0.75
3,4-Dihydroxymandelic acid (DOMA)	–	–	0.80
3,4-Dihydroxyphenylalanine (DOPA)	–	–	0.80
3,4-Dihydroxyphenylethyleneglycol (DHPG)	+	–	0.94
Norepinephrine	+	+	1.71
α-Methyl-3,4-dihydroxyphenylalanine (α-methyldopa)	–	–	1.94
Epinephrine	+	+	2.79
3-Methoxytyrosine	–	–	2.84
α-Methylnorepinephrine	+	+	3.28
3,4-Dihydroxybenzylamine (DHBA)	+	+	4.08
3-Methoxy-4-hydroxyphenylethyleneglycol (MPHG)	–	–	4.18
Normetanephrine	–	–	4.85
N-Methyldopamine	+	+	6.54
Metanephrine	–	–	7.54
Dopamine	+	+	7.69
3,4-Dihydroxyphenylacetic acid (DOPAC)	–	–	7.24
Isoproterenol	+	+	11.42
3-Methoxytyramine	–	–	13.26
α-Methyldopamine	+	+	14.39
Homovanillic acid	–	–	23.78

encountered in normal subjects was not possible due to HPLC interferences. Although results for catecholamines correlated reasonably, significantly less interfering peaks were found with the affinity gel procedure. The correlation between the procedures was: $y=0.91x+1.70$ ($r=0.972$) for NE; $y=0.989x+1.26$ ($r=0.968$) for E; $y=0.856x+17.7$ ($r=0.958$) for DA, with the cation-exchange data treated as the independent variable.

A study of 24-h urine collections from 18 normal subjects and 60 hypertensive subjects, aged between 23 and 76 years, to establish a suitable reference interval, showed a positive, skewed distribution for NE and E but not for DA [13]. The results were subsequently normalised by logarithmic transformation prior to statistical calculation and gave overall ranges of 60–654, 10–202 and 282–2334 nmol per 24 h $\pm$ 2 S.D. for NE, E and DA, respectively.

DISCUSSION

It has been reported that urinary free catecholamine analysis may yield a more reliable and specific diagnostic test for pheochromocytoma than plasma catecholamine levels [14]. Although efficient HPLC procedures are available for catecholamine separation, the purification of urine samples for analysis still pre-

sents difficulties. A two-step sample clean-up is invariably required to minimise the presence of interfering HPLC peaks. This can involve a time-consuming assay using ion-exchange isolation followed by preconcentration on alumina [15]. Recent reports, however, have advocated a single sample clean-up involving boric acid complex formation either from a weak cation exchanger [16] or following extraction with diphenylborate [17]. Despite the increased specificity and analytical recovery achieved by selective complexation with boric acid, the resulting sample clean-up is not always satisfactory since interference from other electroactive compounds is frequently encountered. Furthermore, to achieve adequate resolution of the catecholamines from interfering HPLC peaks requires the use of a high concentration of ion-pairing reagent in the mobile phase. This can subsequently reduce the working life of the reversed-phase column.

Using the immobilised *m*-aminophenylboronic acid gels as a sample clean-up step provides eluates for injection with the minimum presence of interfering peaks and allows the selection of HPLC conditions to enable rapid separation of the catecholamines with a low concentration of ion-pairing reagent. The optimum conditions for achieving resolution and minimum retention time was found to entail the use of 0.25 m*M* OSA and 5% methanol at mobile phase pH values greater than 3.5. As shown in Fig. 2 with different OSA concentrations, alterations in mobile phase at pH 3.5 or greater had little influence on catecholamine retentions. Similar chromatographic data were also obtained with 250 mm × 4.6 mm Hypersil ODS HPLC columns with a methanol concentration of 10%. The estimation of E in patients administered α-methyldopa frequently presents difficulties due to the presence of the metabolite, α-methylnorepinephrine, which can have the same HPLC retention time as E. With the selected HPLC conditions, E is completely separated from α-methylnorepinephrine.

Furthermore, the use of a formate HPLC mobile phase provided enhanced long-term stability of the stationary phase in the presence of the ion-pairing agent and improved the detector linearity range. Operating the HPLC system at a reduced flow-rate during stand by periods further contributed to a steady baseline and reproducible chromatographic response.

Although the prepacked, affinity gels are designed for the estimation of glycosylated haemoglobin in blood, the ability of the boronic acid gels to form complexes with cis-diol groups makes them ideally suited for catecholamine isolation. The results from the study of the isolation properties of the affinity gels suggests that sample isolation resembles that of immobilised boric acid [10]: retention is based on the presence of vicinal hydroxyl groups. However, the further presence of a carboxylic group adjacent to the vicinal hydroxyl group prevents isolation by the boronic acid gel, as exemplified by 3,4-dihydroxymandelic acid and 3,4-dihydroxyphenylacetic acid. Furthermore, compounds possessing a vicinal hydroxyl group but without a primary amino function (such as 3,4-dihydroxyphenylethyleneglycol) are retained but subsequently removed during the washing stages. Desorption from the boronic acid gels is readily achieved by lowering the pH of the gels with acetic acid causing dissociation of the boronic acid–catecholamine complex.

Previous uses of immobilised boric acid for catecholamine isolation have in-

volved acrylamide-based gels [10,18] and have shown batch-to-batch variation in the isolation properties. The affinity gels used in this study consist of a ligand attached to an agarose matrix and as such have shown no variation in batches over a two-year period. Moreover, the stability of the catecholamines in the acetic acid eluates and the use of an isocratic mobile phase makes the procedure suited to the analysis of large batches of samples with an automatic sample injection system. It is a simple, rapid and specific procedure with the benefit of enhanced sensitivity due to the two-fold concentration of the sample volume.

In conclusion, the combination of boronic acid isolation with the selected HPLC conditions of analysis can provide a reliable procedure suited for the routine determination of free catecholamine levels in urine. The sample clean-up step is also economical as affinity gels can be re-used after regeneration at least ten to fifteen times and yield a clean eluate suitable for HPLC analysis with electrochemical detection. Preliminary results also suggest that the eluates are suitable for fluorimetric detection, and the application of the affinity gels to plasma catecholamine isolation is currently being investigated.

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