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CATECHOLAMINE MEASUREMENTS IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION – COMPARISON WITH AN AUTOANALYSER FLUORESCENCE METHOD

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SUMMARY

In order to validate different methods of measuring urinary catecholamines (norepinephrine, epinephrine and dopamine) in humans, methods based on separation of catecholamines using reversed-phase or cation-exchange high-performance liquid chromatography with electrochemical detection were compared with an autoanalyser-based fluorescence method Different methods for pre-chromatography sample purification were also studied For measurements of urinary cate-

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cholamines, the reversed-phase-based chromatographic techniques studied were found to give less reliable results than cation-exchange chromatography, even if one of them (Clin Rep Urine Catecholamine Kit) gave almost as precise estimates The autoanalyser technique yielded good results It is concluded that cation-exchange chromatography with an appropriate sample work-up procedure (a combination of organic solvent extraction and alumina adsorption) is a reliable and accurate method for analyses of urinary catecholamines

INTRODUCTION

Determinations of catecholamines (CAs) in urine have been used extensively to assess sympatho-adrenal activity, e.g in connection with stress research [1] and in clinical conditions, such as pheochromocytoma [2]. Different radioenzymic and fluorimetric methods have been used to assess CAs in urine. More recently high-performance liquid chromatography (HPLC) with electrochemical detection (ED) using either cation-exchange [3–8] or reversed-phase [9–12] columns has gained acceptance However, few attempts to evaluate the urinary assays by directly comparing results have been published [7, 13, 14] Urine contains about 50–100 times more norepinephrine (NE) and epinephrine (E) than does plasma, and even more dopamine (DA). Hence, sensitivity is not a major problem when assaying urinary CAs although specificity may remain a difficulty, as is sometimes the case with plasma CA assays It has been shown that laboratories using different techniques can obtain widely differing estimates of CAs in plasma even when high levels are assayed [15] Since urine is a complex biological matrix with many possibly interfering substances, it can be assumed that problems with lack of specificity may be at least as great with this material For example, our simple and well validated assay for plasma [16] cannot be used to assay CAs in human urine without further modification.

A variety of extraction methods has been used to purify and concentrate urine samples. Adsorption on alumina is a simple and rapid technique for the enrichment and purification of catechols [17], but has seemed inadequate in this respect when using reversed-phase HPLC [12, 18] Combinations of alumina adsorption and separation over C_{18} and silica pre-packed columns [10] or cation-exchange columns [9] have been tried. Organic solvents may be used either to extract interfering compounds [3, 5, 11] or to extract the CAs themselves, e g as diphenylborate–CA complexes [4, 19] from urine. Different analyses may impose different requirements on the sample work-up

The separation step, as well as the work-up procedure, is important to achieve specificity in HPLC assays For CAs, cation-exchange columns may offer a higher degree of selectivity than reversed-phase HPLC [5, 20] The specificity of an HPLC assay can be altered by modifying the mobile phase, thus changing the retention times of CAs relative to possibly interfering substances [20] This is commonly employed in reversed-phase HPLC, but can also be used

with cation-exchange HPLC [5]. Thus the specificity of an assay depends on the proper combination of a suitable work-up procedure and a reliable separation in the assay step.

To evaluate urinary CA assays, we have compared assay results obtained with various HPLC techniques and sample work-up procedures with those obtained with a fluorimetric assay [21], which has been used extensively.

EXPERIMENTAL

Reagents

Water was purified by triple distillation in glass, with potassium permanganate (1 g/l) and sodium hydroxide (1 g/l) added in the last step. Tetrahydrofuran and hexane were both of HPLC grade (Rathburn, Walkerburn, U.K.). Ethyl acetate, perchloric acid, sodium metabisulphite and buffer substances were all purchased from E. Merck (Darmstadt, F.R.G.). Aluminium oxide was purchased from BDH (Poole, U.K.) and activated according to Anton and Sayre [17] C_{18} Sep-Pak and Silica Sep-Pak were purchased from Millipore/Waters Assoc. (Milford, MA, U.S.A.). Stock solutions (10 m*M*) of NE, E, DA (all from Sigma, St. Louis, MO, U.S.A.), α-methyldopamine (Merck, Sharp & Dohme, Rahway, NJ, U.S.A.) and 3,4-dihydroxybenzylamine (DHBA; Aldrich, Milwaukee, WI, U.S.A.) were made up in 0.1 m*M* perchloric acid with sodium metabisulphite and stored in aliquots at $-80°C$. The Clin Rep Urine Catecholamine Kit (Recipe Pharma Vertriebs, Munich, F.R.G.) contains reagents for sample preparation [5 m*M* hydrochloric acid, 0.1% EDTA, 0.5 m*M* sodium hydroxide, 4% boric acid and DHBA (10 ng/μl ($\approx$70 μM) as internal standard)] and standards [NE 10 pg/μl ($\approx$59 n*M*), E 6 pg/μl ($\approx$33 n*M*), DHBA 10 pg/μl ($\approx$70 n*M*) and DA 40 pg/μl ($\approx$250 n*M*)]. The mobile phase for the HPLC column is delivered with the kit. For the autoanalyser fluorescence (AF) method, standards of 0.1 μg/ml E or NE were obtained by diluting stock standards of 100 μg/ml 1:1000 in 0.25 *M* acetic acid.

HPLC equipment

The mobile phase was delivered by Constametric I or III pumps (Laboratory Data Control, Riviera Beach, FL, U.S.A.) equipped with extra pulse dampeners (Touzart-Matignon, Vitry sur Seine, France). As sample injectors we used Rheodyne 7125 (Rheodyne, Berkeley, CA, U.S.A.) or Waters U6K (Millipore/Waters Assoc.). The stainless-steel columns were packed with Nucleosil 5 SA, 10 SA, 5 C_{18} or 10 C_{18} (Macherey-Nagel, Düren, F.R.G.). The 5 SA column, i.e. the one most frequently used, was 20 cm$\times$4 mm I.D.; the others were 25–30 cm$\times$4 mm I.D. The Clin Rep Urine Catecholamine Kit included a prepacked 15-cm reversed-phase column from Waters Assoc. As electrochemical detectors, either LC-4/LC-4A (Bioanalytical Systems, W. Lafayette, IN,

U S A) or the Waters M460 have been used The detectors are amperometric and were operated at +0 6 V utilizing Ag/AgCl reference electrodes and glassy carbon working electrodes Various two-channel strip chart recorders were used

Equipment used in the AF assay

The autoanalyser system consisted of the following parts, all from Technicon (Tarrytown, NY, U S A) a sampler II, a proportioning pump II, two manifolds (one for E and one for NE) and a fluorimeter II (FM II) Depending on the excitation spectra of the fluorophores from the oxidation of the CAs, one of two primary interference filters was used For E, 4410 ± 130 Å (DAL 025403) from Scott (Jena Mainz), and for NE, 4025 ± 25 Å (Type 14-67-2) from Baird Atomic (Cambridge, MA, U S A) In both cases a yellow glass filter (Type CS-3-70) from Corning absorbing light with wavelengths <5100 Å (cut-off wavelength) was used

Sample preparation

Freshly voided urine from healthy male subjects was collected under various activities, in order to provide a wide span of CAs, and adjusted to pH 3 0 within 1 h by the addition of 6 *M* hydrochloric acid The volume of each sample was recorded and then divided into several aliquots The samples were kept frozen at −20°C until analysed Spiked urine samples were prepared by adding 4 nmol of NE, 1 6 nmol of E and 8 nmol of DA from stock solutions to 10 ml of urine sample

A Preparation for analysis by cation-exchange HPLC

1 Cation-exchange followed by alumina extraction. The samples were prepared according to Riggin and Kissinger [9] with minor modifications Cation-exchange columns were prepared by rinsing cation-exchange resin (Bio-Rex 70, Bio-Rad Labs , Richmond, CA, U S A) with successive volumes of 3 *M* HCl, 3 *M* sodium hydroxide, 3 *M* acetic acid and 0 1 *M* pH 6.5 sodium phosphate buffer The pH was adjusted to 6.5 during the last wash if necessary Plastic isolation columns (Bio-Rad Labs) were loaded with resin and washed with 2 ml of phosphate buffer just before use Then 100 pmol of DHBA in 200 μl of 0 1% PCA were added to 5 ml of urine and this was mixed with 15 ml of 0 1 *M* phosphate buffer (pH 7) containing 1% EDTA The pH was checked and when necessary adjusted to 7 0 The entire sample was carefully poured on to the column After complete drainage the column was washed with 10 ml of distilled water, followed by 1 5 ml of 0 7 *M* sulphuric acid The CAs were then eluted with 4 ml of 1 4 *M* ammonium sulphate into vials containing 50 mg of alumina and 30 μl of sodium metabisulphite Next, 3 ml of 1 *M* Tris (pH 8 6) with 2% EDTA were added After 15 min of vortex-mixing to adsorb the CAs,

the alumina was carefully rinsed with cold water and finally the CAs were desorbed into 200 μl of 0.1 *M* PCA, which was removed and stored at $-20°C$ until analysed

2 *Solvent clean-up with ethyl acetate–hexane followed by alumina extraction* A clean-up procedure according to Kissinger et al [3] and Eriksson et al [5] was used. This involved 2 ml of thawed and centrifuged (2 min, 1000 *g* at 4°C) urine samples, spiked urine samples or standard mixes, to which α-methyl dopamine was added as internal standard (concentration in the urine sample: 0.29 μM) The mixture was shaken by hand with 4 ml of ethyl acetate for 2–4 s, then centrifuged at 1000 *g* for 2–3 min, after which the organic solvent was aspirated and discarded. Another 4 ml of ethyl acetate were added and the procedure was repeated Lastly, 2 ml of hexane was added, the sample was shaken and centrifuged, and the organic phase was again thoroughly aspirated The aqueous phase (0 5 ml) was then further purified by adsorption on alumina as described under *A1*

B Preparation for analysis by reversed-phase HPLC

1 Cation-exchange purification followed by alumina extraction The samples were prepared for chromatography as described under *A1*

2 Preparation for analysis by the Clin Rep Urine Catecholamine Kit Using reagents supplied in the kit, 3 ml of urine were prepared by a sample clean-up procede using a weak cation-exchange column, which was eluted with boric acid according to instructions from the company DHBA was used as internal standard

C Preparation for analysis by the AF assay

A 25-ml volume of acidified urine was thawed and filtered through filter paper, 0 5 g 1% EDTA was added and the pH was adjusted to 8 3 $\pm$ 0 2 with 1 *M* sodium hydroxide The sample was then immediately poured onto an alumina column and passed through it by gravity The column was then washed with 10 ml of 1% EDTA followed by 150 ml of water (suction by a water beam vacuum pump) The CAs were eluted with 15 ml of 0 25 *M* acetic acid and kept frozen ($-20°C$) until analysed For further details, see Andersson et al. [21]

Analysis

A Cation-exchange HPLC-ED

The detector setting was +0 60 V and the sensitivity ranges 0 2–0 5 nA/V (BAS) or 0 5 nA/10 mV (Waters) A 50–100 μl volume of the CA eluate was injected onto the chromatographic system (Nucleosil 5SA or 10SA column) The concentration of each CA was estimated by peak-height measurements and adjusted for losses during sample work-up by the recovery of the internal standard The mobile phase was a pH 5 2 citrate buffer (0 39%, w/v, sodium

hydroxide and 1.10%, w/v, citric acid monohydrate), containing 3% tetrahydrofuran for analysis of samples prepared as described under *Solvent clean-up with ethyl acetate–hexane followed by alumina extraction* (*A2*). For samples prepared as described under *Cation-exchange followed by alumina extraction* (*A1*), an acetate–citrate buffer (pH 5.2) containing 5.75 g/l citric acid monohydrate, 6.80 g/l sodium acetate·$3H_2O$, 1.05 ml/l glacial acetic acid, and 2.40 g/l sodium hydroxide was used.

B. Reversed-phase HPLC–ED

A 25–50 μl aliquot of the eluate, prepared a described under *B1* was injected into a chromatographic system with a Nucleosil 5 or 10 C_{18} column and an electrochemical detector (BAS) equipped with TL-5A glassy carbon thin-layer electrode cells. The detector setting was + 0.60 V and the sensitivity range was 0.2–0.5 nA/V (BAS). The mobile phase was made by mixing and filtering 300 ml of 0.1 *M* citric acid and 160 ml 0.1 *M* Na_2HPO_4. Sodium octylsulphate (final concentration 1 m*M*) was added to the mobile phase. The CA concentrations were estimated as for cation exchange. For analyses with the Clin Rep Urine Catecholamine Kit, 4 μl of the eluate were injected onto the chromatographic system according to instructions in the kit, using the column supplied with the kit. The CA concentrations were estimated as before.

C. AF assay

Each eluate was analysed in the autoanalyser system in four different ways: twice through the E manifold – with and without (for blanks) the reducing agent ascorbic acid; and twice through the NE manifold – with and without the reducing agent thioglycolic acid. Standard solutions of E and NE were analysed in the same way as the actual sample.

The fluorescence readings from the four different analyses of the eluates, as well as those from the standards, were determined. The results from the analyses of the blanks were subtracted from those of the corresponding analyses. The concentrations of the CAs could then be derived from a system of equations (for further details, see ref. 21).

RESULTS

A. Cation-exchange HPLC

1. Sample preparation with cation-exchange columns and alumina

With this technique the overall recovery of the internal standard was 74 ± 11 (S.D.)%. There was a good correlation ($r = 0.97$; $n = 42$) for NE between the AF method (x) and this method (y), with a line of regression of $y = 1.03x - 14.8$.

This sample clean-up was not sufficient for measurements of E with cation-exchange HPLC, as interfering substances co-chromatographed with E

2 Sample preparation using solvent clean-up with ethyl acetate–hexane followed by alumina

This sample preparation procedure yielded reasonably good overall recovery (50 ± 6 (S D) %) of the internal standard and resulted in markedly reduced problems with interfering peaks when analysed by cation-exchange HPLC (see Fig 1) There was no interfering peak with the same retention time as that of the internal standard α-methyl dopamine The recoveries of added CAs (relative to that of the internal standard) were 99 ± 3% for NE, 97 ± 3% for E, and 101 ± 2% for DA when 20 spiked urine samples were analysed with α-methyl dopamine as internal standard In one experiment the recoveries of CA standards in distilled water (NE $4 \cdot 10^{-7}$, E 1 $6 \cdot 10^{-7}$ and DA $8 \cdot 10^{-7}$ *M*), and the influence of different pH and ionic strength of the aqueous phase on the recoveries were studied Hydrochloric acid (0 25 m*M*) and sodium hydroxide (0 25 m*M*) were added to the aqueous phase to titrate four different pH (pH 2 08, 3 10, 4 20 and 5 59) Sodium chloride was added to obtain different molarities (1 0, 0 5, 0.15, 0.075 and 0 m*M*) The pH and ionic strength in these ranges did not influence recoveries Comparisons of NE and E concentrations

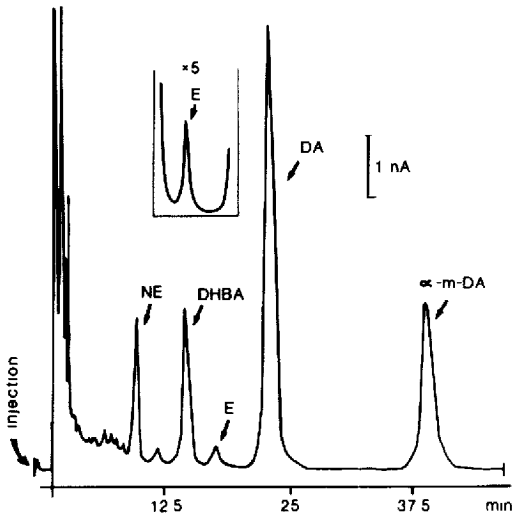

Fig 1 Chromatogram from a urine sample prepared using the sample work-up with ethyl acetate-hexane followed by alumina extraction, 50 μl of a 200-μl eluate was injected, corresponding to 125 μl of urine DHBA and α-methyl dopamine (α-m-DA) were added as internal standards The recovery of DHBA was 50% and the recovery of m-DA was 48% Calculated CA concentrations were 560 n*M* for NE, 130 n*M* for E and 2 48 μ*M* for DA The sample clean-up considerably decreased the presence of interfering peaks, especially in the vicinity of NE

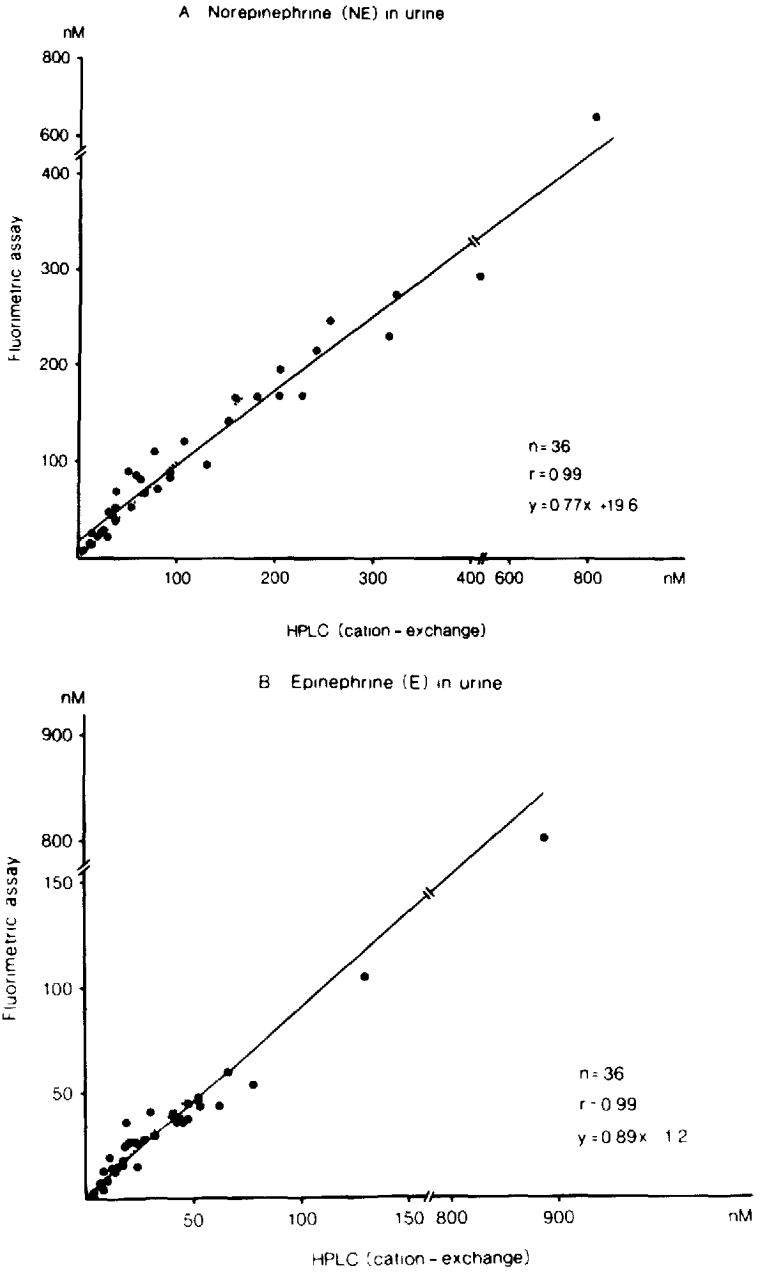

Fig 2 Comparisons between norepinephrine (A) and epinephrine (B) concentrations assessed by cation-exchange HPLC (x-axis) and an AF assay (y-axis), $n=36$

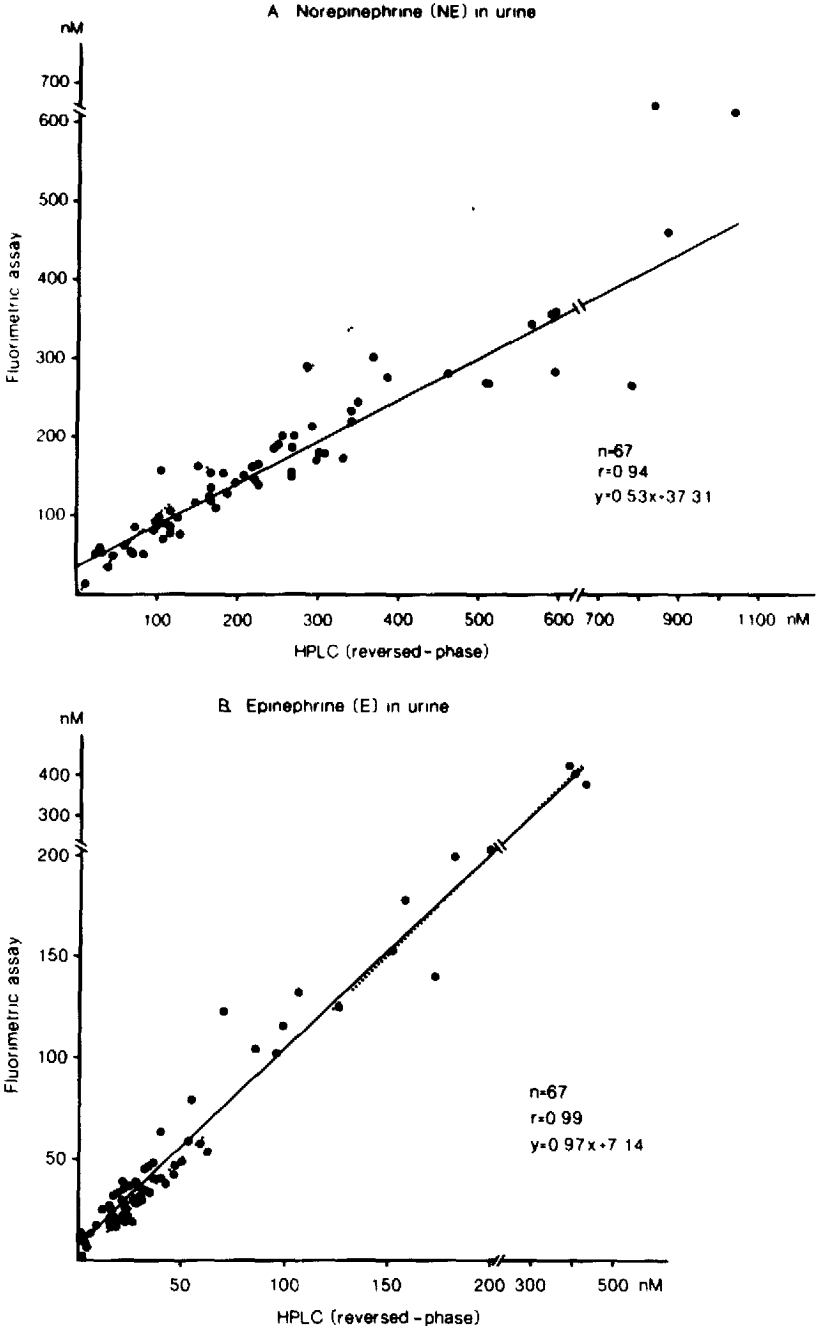

Fig 3 Comparisons between norepinephrine (A) and epinephrine (B) concentrations in urine assessed by reversed-phase HPLC (sample preparation with cation-exchange columns and alumina) (x-axis) and an AF assay (y-axis), $n=67$

in urine samples ($n=36$) assessed by modified cation-exchange HPLC and the AF assay yielded excellent agreement (see Fig. 2)

B Reversed-phase HPLC

1 Sample preparation with cation-exchange columns and alumina

The NE values were higher when analysed by reversed-phase HPLC, especially at concentrations over 150 n*M*, whereas E levels were similar when analysed by reversed-phase HPLC and the AF assay (Fig 3) When the same eluates were analysed on cation-exchange columns (without tetrahydrofuran in the mobile phase), NE values agreed with those obtained with the AF method, whereas E peaks were contaminated (see *A1*)

2 Clin Rep Urine Catecholamine Kit

Comparisons of urinary CA levels obtained with the Clin Rep Urine Catecholamine Kit (y) and the cation-exchange HPLC (x) are shown in Fig. 4 NE concentrations were almost identical when assessed by these two methods, and there was good agreement also for DA concentrations With one exception, E levels also showed good agreement ($r=0\,99$, $n=35$, Fig 4) In the remaining sample the E peak was followed by a contaminating peak not baseline-separated from the E peak when assessed by the Clin Rep Urine Catecholamine Kit The calculated E concentration was 807 n*M* The corresponding E con-

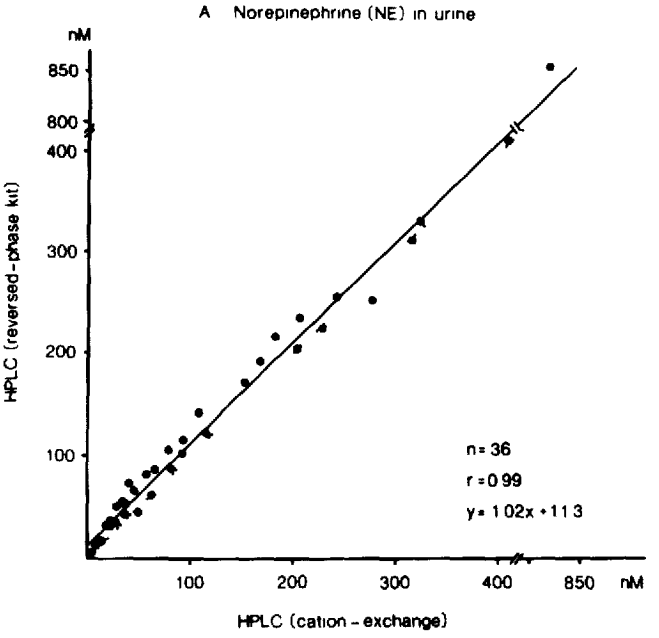

Fig 4

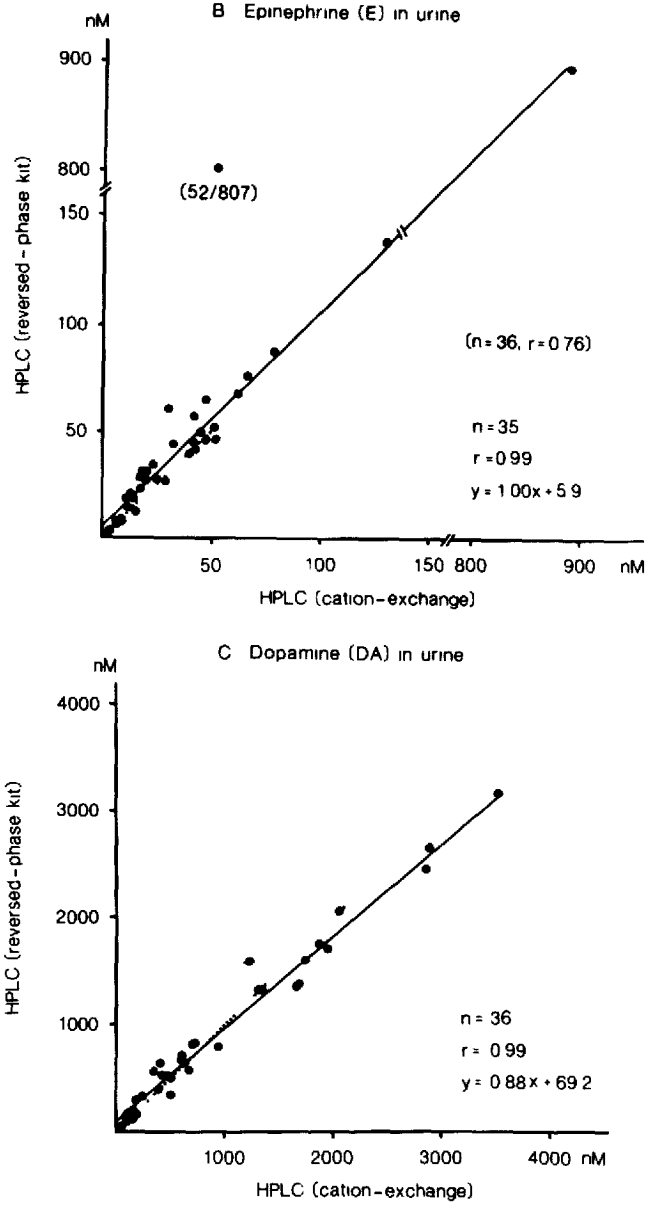

Fig 4 Comparisons between norepinephrine (A), epinephrine (B) and dopamine (C) concentrations in urine estimated by analysis on cation-exchange HPLC (x-axis) and reversed-phase HPLC (preparation according to the Clin Rep Urine Catecholamine Kit, y-axis), $n=36$ The comparison of E concentrations (B) comprises 35 samples since one sample (shown in parentheses) differed considerably and was not included in the calculation of the line of regression (see text)

centration was 52 nM when assessed by cation-exchange HPLC Thus, the correlation coefficient was low (r=0 76) and the line of regression was y=1 00x+26 9 for E concentrations when assessed by these two methods, if all urine samples (n=36) were included

The CA concentrations assessed by the Clin Rep Urine Catecholamine Kit (x) were compared with the AF assay (y) The lines of regression were y=0 75x+11 1 (r=0 99, n=36) for NE and y=0 51x+8 9 (r=0 76, n=36) for E (no figure shown) The E concentration that differed greatly when assessed by the Clin Rep Urine Catecholamine Kit and the cation-exchange HPLC was 46 nM when assessed by the AF assay, i e almost identical with the concentration obtained with cation-exchange HPLC (52 nM) After exclusion of this sample, the correlation coefficient was r=0 99 and the line of regression was y=0 89x−3 8 (n=35) As the AF assay and the cation-exchange HPLC method both gave a similar low value, the high E value obtained with the Clin Rep Urine Catecholamine Kit was probably due to the contaminating peak not baseline-separated from E

DISCUSSION

The main findings of this study were (i) that a method using organic solvent clean-up with ethyl acetate and hexane followed by alumina adsorption in combination with analysis using cation-exchange HPLC [5] provided clean chromatograms and an acceptable overall recovery that was equal for all three CAs (NE, E and DA) (ii) that assessment of CAs in urine by this modified cation-exchange HPLC method seems more reliable and accurate than by the two reversed-phase HPLC methods used, (iii) that the fluorimetric assay gives good estimates of NE and, in particular, E in urine The second statement is based on findings of an interference with E in one sample with the Clin Rep Urine Catecholamine Kit, and that the reversed-phase HPLC method described by Riggin and Kissinger [9] clearly overestimated NE levels at intermediate and high concentrations in urine

With regard to sample clean-up, the present and previously published comparisons and descriptions of methods used to estimate CAs in human urine have shown that alumina adsorption alone is insufficient for analysis on reversed-phase [12, 18] or cation-exchange [7] HPLC with ED Sample clean-up on weak cation-exchange columns alone was also found to be inefficient when samples were analysed by cation-exchange HPLC with ED [7] When using a combination of weak cation-exchange columns and alumina, we obtained accurate values for NE, as did others [7], but we were unable to estimate E levels owing to interfering compounds

In addition to the work-up procedures presented in this study, we also tried sample preparation with solvent extraction of diphenylborate–CA complexes [4], which has been used successfully by others [13], however, we did not

obtain sufficiently clean chromatograms with this procedure With additional extraction over alumina, an unacceptably low overall recovery (25–30%) of CA standards and internal standards was obtained (unpublished results). Using the method of Goldstein [10], i e. sample clean-up on C_{18} and Silica Sep-Pak cartridges and alumina extraction, we found a considerable decrease in the recovery of CA standards and internal standards with increasing polarity of the compound extracted (unpublished results) For example, only one third of added DA was recovered, when compared with the recovery of added NE Tests of the three different steps of the method separately revealed that the difference in recovery most probably occurred during the C_{18} Sep-Pak step

Solvent clean-up with ethyl acetate–hexane followed by alumina adsorption proved, in our hands, to be the purification procedure best suited for analysis of urine samples by cation-exchange HPLC– ED This is, however, probably not the case for reversed-phase HPLC–ED, as interferences with E have been observed using similar purification procedures [12, 22]

Both of the reversed-phase HPLC methods used by us resulted in some problems with interferences or poor agreement with other assay techniques The Clin Rep Urine Catecholamine Kit yielded results that generally agreed well with the levels of CAs estimated by the AF and cation-exchange HPLC methods, but showed an interference with E leading to an unacceptable overestimation of this CA in one out of 36 samples In our hands, the method of Rigging and Kissinger [9] yielded overestimations of NE levels when compared with both the AF method and cation-exchange HPLC; Weicker et al [14] found a good correlation for NE, but a low correlation for E when compared with a fluorimetric assay

To our knowledge, this is the first study to validate a cation-exchange HPLC technique for analysis of urinary NE, E and DA by a direct comparison of results obtained with this and other methods As mentioned, the purification procedure is of great importance also when analysing CAs by cation-exchange HPLC–ED Thus, the simple alumina extraction technique, which gives reliable results for CAs in plasma when using cation-exchange HPLC [15, 23], was not sufficient for measurements in human urine. Addition of a solvent clean-up step prior to chromatography, and of tetrahydrofuran to the mobile phase, resulted in clean chromatograms in agreement with previous results [5]

The present results also show that the AF method, which has been used extensively for some 25 years [24], is still a reliable and sensitive technique for quantitative differential analysis of urinary E and NE. The method distinguishes the two CAs at least as well as, and in some cases better than HPLC techniques. The AF method of Andersson et al. [21] gives reliable values of NE and E in urine but does not determine DA

With regard to HPLC-based analyses, the modified cation-exchange method,

in combination with appropriate sample work-up procedure, gave accurate results for NE, E and DA as judged by comparison with other techniques.

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