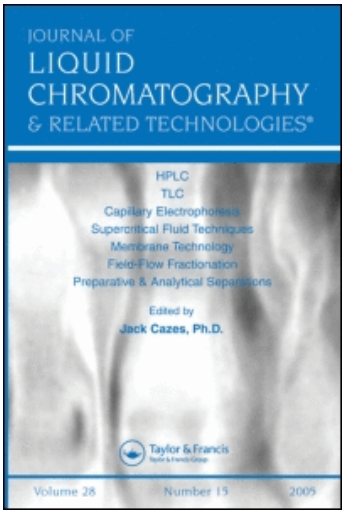

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Solid Phase Reactors as an Analytical Tool in the Determination of Urinary Noradrenaline and Adrenaline

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To cite this Article Rüter, J. , Kurz, U. P. and Neidhart, B.(1985) 'Solid Phase Reactors as an Analytical Tool in the Determination of Urinary Noradrenaline and Adrenaline', Journal of Liquid Chromatography & Related Technologies, 8: 13, 2475 — 2496
To link to this Article: DOI: 10.1080/01483918508076582
URL: http://dx.doi.org/10.1080/01483918508076582

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(13), 2475-2496 (1985)

SOLID PHASE REACTORS AS AN ANALYTICAL TOOL IN THE DETERMINATION OF URINARY NORADRENALINE AND ADRENALINE

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ABSTRACT

The possibility of performing the oxidation of the catecholamines noradrenaline and adrenaline inside a PbO_2- or a MnO_2- solid phase reactor (SPR), which is integrated in the chemical reaction detector of the trihydroxyindole (THI) method is demonstrated. The adaptation of the SPR to the chromatographic conditions and the optimization of the different reaction parameters is described. A comparison of the two SPRs to the ferricyanide oxidation in the homogenous system is made with respect to accuracy, sensitivity and precision of the results. The new SPR-technique enables simplification and low cost running of the THI-system in routine analysis of urinary catecholamines.

INTRODUCTION

The physiological and pharmacological importance of the biogenic amines noradrenaline (NA) and adrenaline

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(A) has promoted a lot of research in the analytical determination of these neurotransmitters. Spectrophotometric (1) and fluorometric (2-15) methods are used as well as electrochemical (16-21) and radioenzymatical (22-28) methods. Separation is performed mainly by high-performance liquid chromatography and sometimes by gas chromatography or thin-layer chromatography (15). Because of the instability of NA and A with respect to oxidation (29) and enzymatic decomposition, the isolation of the catecholamines (CA) from the matrix (urine or blood) is a problem. Cation-exchange procedures as well as adsorption onto aluminum oxide or boric acid gel followed by acid elution have been used successfully (14,15). In our laboratory the trihydroxyindol-method (THI) in combination with Al_2O_3 extraction and HPLC is mainly used. The measurements are performed online in a chemical reaction detector. The complete analytical procedure has been described elsewhere (13). Inside the chemical reaction system the first step within the procedure of forming the fluorescent trihydroxyindoles is the oxidation of the catecholamines to the respective adrenochromes (4). For this step different oxidants like ferricyanide, Cu^{2+}, Fe^{3+} or MnO_2 can be used. In the case of solid reagents like MnO_2 time consuming batch procedures were employed.

Recently we have described a solid phase reactor (SPR) for the online oxidation of manganese (30) and chromium. This SPR is filled with solid PbO_2 by which the sample is oxidized when passing the reactor column. The reaction conditions are influenced by different parameters like pH, temperature, and reactor dimensions etc. (31). Other authors (32, 33) have used similar SPRs with other packing materials for different reactions in post column detection.

In this paper we demonstrate the possibility of performing the oxidation of catecholamines (NA + A) inside a solid phase reactor, integrated in the conventional chemical reaction detector of the THI-method. Lead dioxide and manganese dioxide are proven to be suitable reactor packing materials. The reaction conditions in the chemical reaction system are adapted to the conditions inside the SPR with the aim of getting an optimum oxidation. A comparison between the two SPRs and the ferricyanide oxidation is made with respect to sensitivity and precision in order to allow the judgement whether the use of an SPR enables simplification and low cost running of the THIsystem. Furthermore, the SPRs are tested in the routine analysis of urine samples.

EXPERIMENTAL

Reagents and Solutions:

The chemicals and solutions used for sample pretreatment are described elsewhere with the exception that thymolblue (Riedel-De Haen, Seelze-Hannover) was used as indicator: 1 g/l 20 % ethanol (29).

noradrenaline: L-noradrenaline bitartrate, pharm. (Serva, Heidelberg, FRG)

adrenaline: L-adrenaline bitartrate, B grade (Calbiochem, Calif. USA)

2-mercaptoethanol: puriss (Serva, Heidelberg, FRG)

All other chemicals were of analytical grade and purchased form Merck (Darmstadt, FRG)

- mobile phase in HPLC: 0.05 M potassium perchlorate
  + 250 ul aqueous copper acetate solution (3 g/100 ml H_2O) in 1000 ml of mobile phase
  + 1 g sodium acetate in 100 ml of mobile phase
  + so much acetic acid to adjust the mobile phase to a pH = 4.45

- reducing solution: 266 g sodium hydroxyde
  + 13.4 g sodium sulfite (anhydrous)
  + 9 ml 2-mercaptoethanol
  in 1000 ml bidistilled water

- lead dioxide reactor: preparation described elsewhere (30)

- manganese dioxide reactor: 75.3 g manganese nitrate are dissolved in 500 ml bidistilled water. In this solution 50 g silica gel (18-35 mesh, Macherey-Nagel Co., Düren, F.R.G.) are suspended by vigorous stirring; to this suspension a solution of 31.6 g potassium permanganate in 500 ml bidistilled water is slowly added. A part of the precipitating MnO_2 is adsorbed by the suspended silica gel. After 30 minutes of stirring the silica gel is filtered off

through a 500 µm sieve and washed until no permanganate colouring is left. The final product is dried in a desiccator.

Apparatus

HPLC pump:	Waters model M-45 (Waters GmbH, D-6240 Königstein/Taunus, FRG)
Switching valve:	Latek-7010 (Latek, D-6900 Heidelberg, FRG)
Column:	if not otherwise stated, self-packed Nucleosil 5-C_{18} (SS 250/ 1/4" /4,6) (Macherey-Nagel Co., D-5160 Düren, FRG)
Pump tubing and accessories:	Biotechnik GmbH (D-2000 Hamburg, FRG)
Thermostat:	Immersion thermostat T 51 (Heidolph Elektro KG, D-8420 Kelheim, FRG)
PTFE-tubing and accessories:	Latek (D-6900 Heidelberg, FRG)
Fluorometric detector:	Shimadzu Fluorescence Spectromonitor RF-530 with 12 µl flow cell (Shimadzu, D-4000 Düsseldorf)
Recorder:	Corning recorder 840 (IMA Analysentechnik, D-6300 Giessen, FRG)
pH-meter:	Digital-pH meter CG 820, Schott-Geräte GmbH, D-6238 Hofheim a. Ts.
SPR-reactor column:	made of stainless steel tubing or glass tubing (Omnifit)

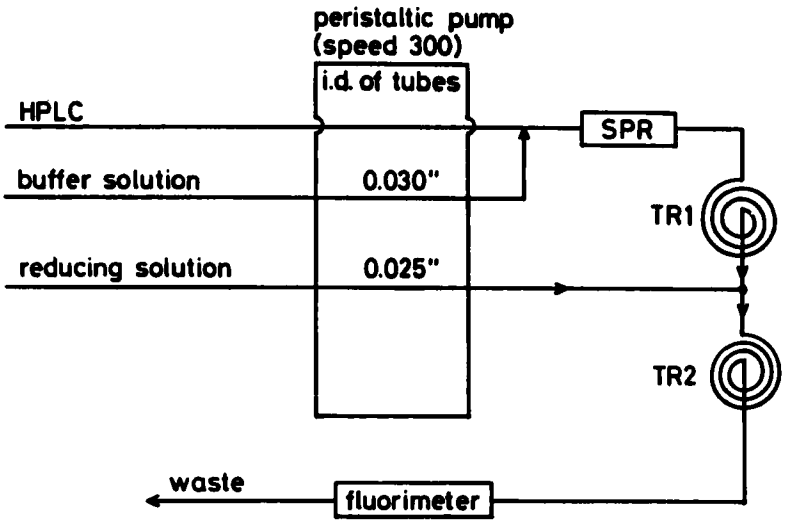

FIGURE 1. Scheme of the heterogeneous chemical reaction detector for NA and A

SPR: Solid Phase Reactor (PbO_2 or MnO_2)
TR1: Teflon Reactor (l = 3.5 m, i.D. = 0.8 mm)
TR2: Teflon Reactor (l = 2.0 m, i.D. = 0.8 mm)

Method

The experimental set-up is shown in Fig. 1. After pretreatment of the samples a 250 µl aliquot of the eluate is injected onto the analytical column. The separation takes place at room temperature and at a flow rate of the mobile phase of 1 ml/min resulting in a pressure drop of 1800 psi of the Nucleosil 5-C_{18} column. The oxidation of the catecholamines noradrenaline and adrenaline to the respective adrenochromes takes place inside the solid phase reactor coupled close behind the analytical column. The optimize dimensions are:

- PbO_2-SPR length = 50 mm i.d. = 3 mm glass column; particle size = 18-35 mesh
- MnO_2-SPR length = 50 mm i.d. = 2,1 mm stainless steel; particle size = 18-35 mesh

In both cases the SPR is kept at room temperature. The alkaline rearrangement takes place at 30°C forming the fluorescent trihydroxyindoles. The measurements are performed at λ ex = 400 nm and λ em = 500 nm. The fluorometer adjustements are: sensitivity "H", range = 1. The recorder voltage is 5 mV and the recorder speed is 5 mm/min. The dimensions of the reaction coils of the homogenous part of the chemical reaction detector are: First PTFE-coil (TR1): l = 3.5 m, i.d. = 0.8 mm; second PTFE-coil (TR2): l = 2 m, i.d. = 0.8 mm.

RESULTS AND DISCUSSION

Although the chemical reaction detector which is used in our laboratory for the determination of urinary NA and A works without any problems in routine analysis (13) we reduced the equipment, the consumption of solvents, and manpower by introducing a solid phase reactor into the system. The oxidation of NA and A now takes place inside the SPR instead of mixing the sample with ferricyanide solution. MnO_2 as oxidant has been used earlier (4) by other authors, but not as an integrated part of the chromatographic system; whereas lead dioxide has not been used so far in catecholamine oxidation.

In the first step of simplification, the all homogeneous chemical reaction system as shown in Fig. 2 was varied by simply leaving the K_3 $(Fe(CN)_6)$ out of the buffered oxidizing solution. The PbO_2- SPR was

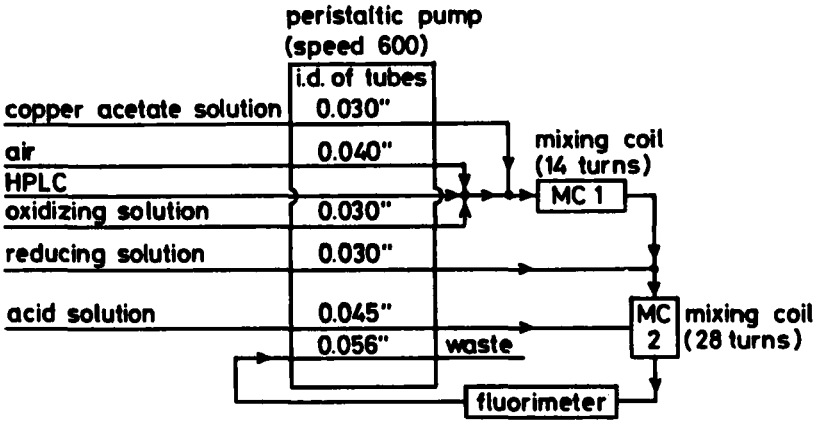

FIGURE 2. Scheme of the all homogeneous chemical reaction detector for NA and A

integrated in the reaction system directly after the inlet of the copper acetate solution. Furthermore, the glass reaction coils (MC 1 and MC 2) were replaced by selfknitted and twisted PTFE capillaries (0.8 mm i.d.). MC 1 was replaced by a 4 m PTFE-reactor. MC 2 was replaced by two PTFE-reactors: one (3 m long) after the inlet of the reducing solution and the other one (3m long) after the inlet of the acid solution. Furthermore, the air tube was removed, because SPRs cannot be used in air segmented systems. The results of CA measurements with this arrangement are given in Fig. 3.

For these measurements a Chromosorb RP-18 column (7 μm, 250 x 4mm) was used together with 0.1 M perchloric acid as mobile phase. Fig. 3 shows that the lead-dioxide SPR is suitable for the continuous detection of NA and A by the THI-method. A disadvantage of the SPR is the apparent deterioration of the chromatographic resolution due to peak broadening inside the SPR.

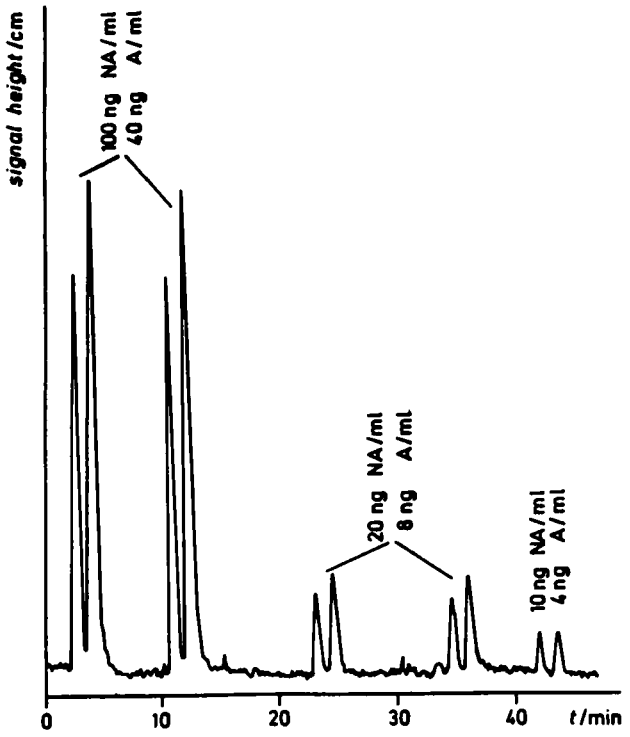

FIGURE 3. Chromatogram of NA and A standard solutions measured with a partially simplified heterogeneous chemical reaction detector

This effect has been discussed in detail by several authors (35, 36). In the given case of NA and A a sufficient chromatographic resolution can only be achieved if the difference in the retention times is at least two minutes.

After these screening experiments, systematic investigations on the influence of different parameters on the oxidation of NA and A were started in order to construct a simpler, with respect to the experimental set-up, chemical reaction detector.

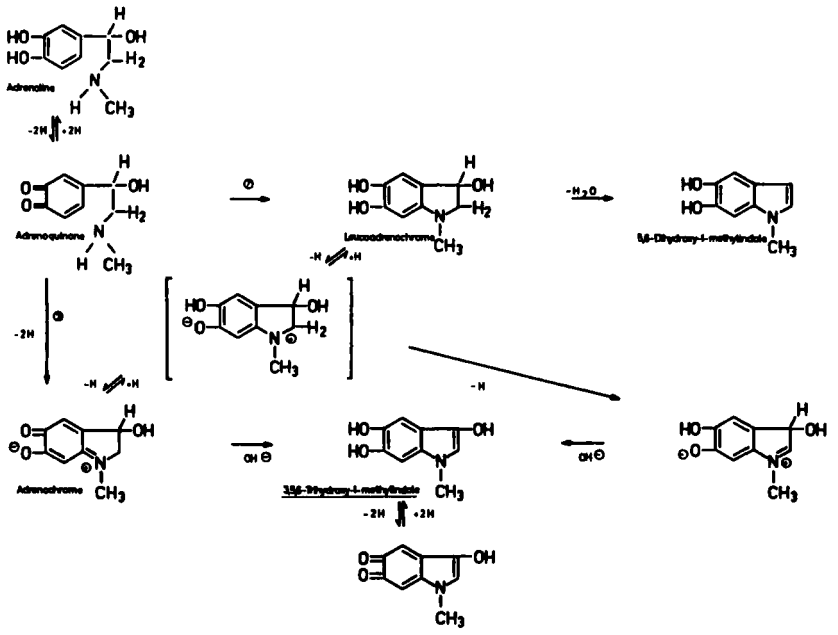

FIGURE 4. Oxidation of A to its trihydroxyindole (analogous for NA)

The controlled oxidative cyclization of the catecholamines to their chrome derivatives and the following intramolecular rearrangement in a strong alkaline solution in the presence of reducing agents (Fig. 4) was first investigated by Lund (9, 10, 37, 38) and Ehrlen (39). These authors showed that the transient fluorescence is due to an uncontrolled oxidation of the fluorophore molecules by dissolved oxygen. The incorporation of a suitable reducing agent in the alkaline solution prevents the undesired oxidation by an excess of oxidant and the dissolved oxygen, thus leading to a stabilization of the fluorophore molecules in the solution. A differentiation between NA and A can be

achieved by running the oxidation procedure at two different pH-values. This is due to the fact that both catecholamines are oxidized over a wide pH range, but with different rate and extend of oxidation for each amine. At lower pH-values (pH 2-4) the formation of the A-fluorophore is favoured whereas at higher pH-values (pH 5-7) the fluorescence of the NA oxidation product is more intensive. A further differentiation between NA and A is possible by choosing different detection wavelengths for the two fluorophores.

Lund (38) used these pH-effects for the differentiated determination of the two catecholamines after oxidation with manganese dioxide. Euler and Floding (5) preferred the oxidation with ferricyanide. In our laboratory, the analysis of NA and A is perfomed by HPLC separation followed by a chemical reaction detector. The pH-value for the oxidation is adjusted in a way that for the NA/A-ratio of 2.5, which is most frequent in urine, the same detector response is obtained.

For the oxidation in a PbO_2 or a MnO_2-reactor the pH-value was systematically varied between 4.0 and 5.0 in order to find the optimum for both amines. The results of these measurements were in good agreement with earlier investigations: at pH 4 the oxidation of A gives very good yields and the signal for NA is low, at pH 5 the reverse result is found. The optimum pH-values for both amines were found to lie between 4.3 and 4.4. Furthermore, it could be shown that both, the temperature of the chemical reaction system and the delay time of the sample in the system, strongly influence the peak heights. For a further simplification of the chemical reaction detector a mobile phase of pH 4.4 for the chromatographic separation of NA and A promised to be advantageous. The profits of this arrangement are firstly a severe reduction of sample dilution because

the same solution is used for both the separation of the catecholamines and their oxidation. This means that the pH value of the mobile phase has no longer to be adapted to the oxidation by adding two separate solutions to the sample stream. Secondly, the SPR can be installed directly behind the separation column. The mobile phase used was a 0.05 M potassium perchlorate solution which was adjusted to pH 4.4 with a 0.2 M acetate buffer solution; to this mixture 250 ul/l of a copper acetate solution (3 g $Cu(CH_3COO)_2$/ 100 ml) was added. Measurements with this experimental set-up showed optimum signal heights at a temperature of 30^oC (all PTFE coils inside a water bath, but not the SPR) and with a length of the first PTFE coil of 350 cm. All separations and measurements with pH adjusted potassium perchlorate as mobile phase had to be performed with a Nucleosil 5-C_{18} packing material because this RP-phase was the only one out of several commercially available C_{18}-materials, which showed no pH-dependent chromatographic resolution for the catecholamines in the pH range from 1.5 to 5.5 (40, 41). In the course of the reorganization of the chemical reaction detector the flow rate of the alkaline solution necessary for the rearrangement of the derivatives could be reduced to 1 ml per minute which again results in less dilution of the sample. Under the above conditions the last step of the THI-method, the reacidification, causes a precipitation in the last reaction coil because of high sample and salt-concentrations. Therefore, the acidification step within the THI-method was eliminated and the fluorophores were measured directly in alkaline solution; this did not cause a serious decrease of the signal heights.

After modifying the chemical reaction detector in the described way it consisted only of the heterogenous

SPR, a first reaction coil, a T-piece for the addition of the reducing alkaline solution, the second reaction coil and the fluorimeter. The experimental set-up is identical with that shown in Fig. 1 except of two variations: the speed of the peristaltic pump is 500 instead of 300 and no buffer solution is added to the eluate of the chromatographic column. PbO_2- as well as MnO_2-SPRs can be used in this chemical reaction detector.

Both SPR-types were used for about 300 measurements without any sign of consumption of the packing material. A comparison between the PbO_2- and the MnO_2-reactor gave similar results concerning the running conditions. The only difference was in the SPR-dimensions. Optimal dimensions of the PbO_2-SPR are a length of 50 mm and an i.d. of 3 mm while a length of 50 mm and an i.d. of 2.1 mm is optimal for the MnO_2-reactor. These results are only valid for the selfmade packing materials. Packing materials with a higher degree of covering for instance would cause a distinct decrease in signal heights, provided that all other parameters are kept constant.

From all measurements it can be clearly concluded that slight variations of pH-value, temperature, or reactor dimensions have a great influence on the signal heights. Therefore, all these parameters have to be controlled carefully.

Finally, a comparison between the all homogeneous reaction system (Fig. 2) and the heterogeneous method using SPRs was performed.

Concerning the consumption of reagents the heterogeneous system is clearly to be preferred to the homogeneous reactor. The heterogeneous system has an overall consumption of 120 ml/h while the "old system" needs about 300 ml/h. This is an important aspect in

routine analysis. With regard to the handling of the systems it is clear that the new system is much easier to operate, even by an unskilled worker. Only two solutions, the mobile phase and the reducing solution, have to be prepared prior to the analysis. The whole apparatus can be operated by one HPLC-pump and a one-channel peristaltic pump, while the homogeneous reaction detector has to be run by a four-channel peristaltic pump with four reagent solutions. Last but not least, the simplified system implies quite less sources of error.

Another important point which had to be considered was the accuracy of the measurements. In order to investigate this point quite a number of urine samples were analysed with both, the homogeneous reaction system on the basis of ferricyanide oxidation and the heterogeneous reaction system with solid supported PbO_2 or MnO_2 as oxidants.

The catecholamine contents of the samples were determined via the standard additions method. Parallel to the analysis of the urine sample, an aliquot was analyzed after the addition of 50 ng NA/ml and 20 ng A/ml urine before sample pretreatment.

The accuracy of the determination is satisfying only in the analysis of A with the MnO_2-reactor. All other correlation coefficients are smaller than 0.9, which was consistent with measurements of standards which showed variations of the mean signal heights attaining 25 % for NA and 15 % for A.

It was mentioned above that the oxidation process with SPRs is very sensitive to pH-variations. Therefore, the pH-value was continuously measured close behind the SPR column with the result that the pH was drifting from the adjusted value to more acidic conditions for about 0.3 pH-units a definite time after having injected a sample. This effect was assumed to be

the consequence of not adapting the sample pretreatment procedure to the varied conditions in the detector. Up to then the sample was dissolved in 0.1 M perchloric acid while the mobile phase consisted of 0.05 M potassium perchlorate which was buffered only weakly. After injection of the acidified sample an acidic zone moved through the system leading to other oxidation conditions on the SPR. In order to minimize this pH-shift the samples were buffered after pretreatment.

Reduction of the sample volume from 250 µl to 100 µl and the pH-adjustment of each sample with 100 µl of 1.0 M NaAc-solution (after clean up) reduced the pH-shift to about 0.05 pH-units. By this measure the precision and the accuracy of the determinations were clearly increased. With this experimental set-up routine analyses of urinary catecholamines were performed for two months at a frequency of 50 analyses per day.

After this period the analytical column had lost its efficiency and a new column was packed, again with Nucleosil 5-C_{18} as packing material. With this new column, however, no satisfying separation of noradrenaline and adrenaline could be achieved at pH-values of 4.4 for the mobile phase. The packing of another analytical column did not change the situation. This was in crass contradiction to earlier experiences, because the Nucleosil 5-C_{18} material had allowed the separation of NA and A at pH-values up to 5.5 for almost five years (13, 40). A possible explanation for this effect is, that the new charges of the RP-material have a different SiO_2-support or the silylation procedure was different compared to that of the earlier charges. This assumption, however, was not confirmed by the supplier (see appendix).

By this surprising development we were forced to go back one step in the simplification procedure. The

new conditions are 0.05 M perchloric acid as mobile phase and for adjustment of the chromatographic eluate to pH 4.4 a sodium acetate solution is pumped into the chemical reaction system just before the SPR. Fig. 1 shows the final experimental set-up. In this system the sample clean-up procedure can remain unchanged, which means no pH-adjustment prior to injection is necessary, as the Al_2O_3-eluate is identical with the mobile phase.

This analytical system was compared to the all homogeneous system in the same way as described before. If a lead dioxide reactor (50 x 1.9 mm) or a manganese dioxide reactor (50 x 3.0 mm) with particle sizes of 18-35 mesh is used, the results for both catecholamines are systematically too high compared to the all homogeneous system. The reason for this is that the calibration curves are linear only up to 50 ng NA/ml and 30 ng A/ml and flatten at higher concentrations. As the catecholamine content of the samples is calculated by the standard additions method with additives of 50 ng NA and 20 ng A per milliliter of the sample and as the standard additions method demands a linear calibration curve, too high values are obtained. A calculation for samples, having additives of 10 ng NA/ml and 4 ng A/ml, which corresponds to the original content of the samples, shows a considerably smaller systematical error (slope of the regression curve = 0.90 for NA and 0.96 for A).

These results were obtained with reactors which were filled with packing materials of 18 - 35 mesh particle size, which corresponds to a degree of covering of 13 % PbO_2 (31). Fig. 5 shows results obtained with a lead dioxide solid phase reactor (20 x 4.6 mm) filled with packing material of 70 - 230 mesh particle size, which corresponds to a degree of covering of 38 %

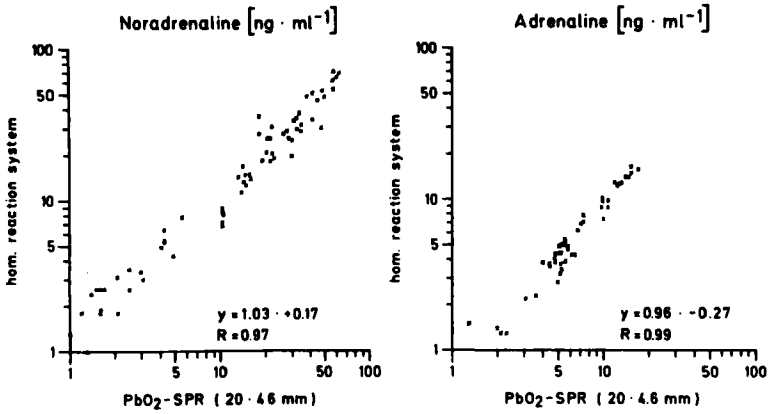

FIGURE 5. Comparison of two detection methods for urinary catecholamines: all homogeneous system / heteregeneous system

PbO_2. With this reactor the calibration curve is linear over the whole range from 0 to 100 ng NA/ml and 0 to 40 ng A/ml. The slopes of 1.03 (NA) and 0.96 (A) and the correlation coefficients of 0.97 (NA) and 0.99 (A) show a good agreement between the values determined with the homogeneous and the heterogeneous chemical system as part of the detector. The accuracy of the all homogeneous system has already been tested by a comparison with other determination methods (40). Fig. 6 shows a section of a chromatogram of routine determinations of urinary catecholamines. The detection limit (3σ-baseline) for the whole course of analysis is 1.0 ng/ml and 0.4 ng/ml for NA and A respectively. The standard deviation (n = 10) is $\pm$ 7 % in the range of 5 - 100 ng/ml.

According to results of the systematical investigation of the lead dioxide reactor, the SPR needs a certain time to bring forth reproducible results (31).

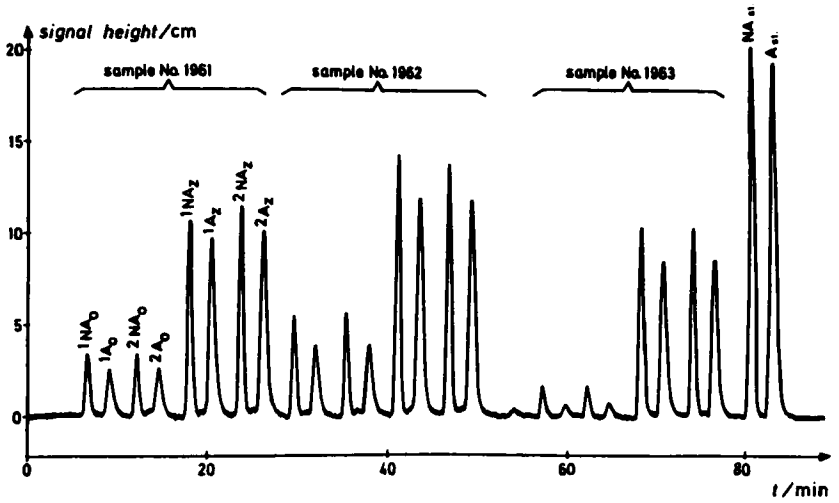

FIGURE 6. Chromatogram of routine determinations of urinary catecholamines using a heterogeneous chemical reaction detector

index st: standard
index o: zero sample
index z: spiked sample

This "running in" phase is completed after about 20 circles of analysis. The exact number differs slightly depending on the reactor dimensions and the particle size of the packing material. The oxidation power of a lead dioxide SPR (50 x 1.9 mm) with a particle size of 18 - 35 mesh shows no deterioration after the determination of 400 samples. A manganese dioxide SPR (50 x 3 mm) with a particle size of 18-35 mesh is consumed after about 350 injections. For routine measurements a lead dioxide reactor (20 x 4.6 mm) with a particle size of 70 - 230 mesh is recommended.

Another advantage of the SPR-oxidation is the suppression of an unassigned third peak which appears in some of the chromatograms of urinary catecholamines

a certain time after the adrenaline signal, if the homogeneous system with ferricyanide oxidation is used (13). The suppression of the unassigned peak is assumed to be the consequence of the different oxidation conditions within the heterogenous reaction system. Thus, because of shorter run-times of the chromatograms, the frequency of analysis can be increased, with a new sample being injected into the system every five minutes.

The experimental set-up described was introduced into routine analysis of urinary catecholamines quite a time ago and still works without any problems.

In the next stage of the investigations the adaptation of the solid phase reactor to the microbore-technique will be examined with the aim to reduce further the consumption of the solvents and to increase the sensitivity. If this is successful, the application of the new technique to the analysis of plasma catecholamines should also be possible.

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APPENDIX

Very recent experiments with Nucleosil 5-C_{18} showed that after a special treatment of this material the separation of NA and A becomes possible even at pH values of up to 5.5. If this change in the separation behaviour turns out to be reproducible, the most simple chemical reaction system of those described in this paper would again be favoured. In that case the question has still to be answered, whether other commercially available RP-phases can be treated in a similar way, in order to qualify for a pH independant CA-separation. These experiments are now under investigation (41).