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# HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF REPROTEROL IN PLASMA USING ON-LINE TRACE ENRICHMENT AND AMPEROMETRIC DETECTION WITH A ROTATING WORKING ELECTRODE

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## SUMMARY

A method is described to determine nanogram quantities of reproterol in plasma. It consists of deproteinization of the plasma samples, on-line trace enrichment, and liquid chromatographic separation of the compounds brought on the analytical column, coupled with amperometric detection. Reliable quantitation can be done down to levels of 1 ng/ml.

## INTRODUCTION

Reproterol (Bronchospasmin® in G.F.R.; Bronchodil® in Great Britain) is a new drug in the treatment of chronic aspecific respiratory affections. It has $\beta_2$-sympathicomimetic activity [1, 2] and its structure is depicted in Fig. 1.

So far, information on the fate of this drug in the body has only been obtained from experiments involving radioactively labeled material [3]. Therapeutically effective oral doses are reported to be 20 mg, three times daily [4, 5] which may lead to plasma concentrations in the order of 3—30 ng/ml or less, since total radioactivity was measured [3].

To investigate its pharmacokinetic properties in man in detail, a suitable method to measure low concentrations of unlabeled reproterol in plasma was necessary. Until now, only analytical methods consisting of sample clean-up, gas chromatography and mass spectrometric (GC—MS) detection have been used to investigate the pharmacokinetic properties of $\beta_2$-sympathicomimetic

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drugs like terbutaline and salbutamol [6—9]. The reason for this was that only GC—MS methods seemed to offer sufficient sensitivity and selectivity to measure therapeutic levels of these drugs in plasma.

From 1973 on, investigations have been published in which catecholamines were measured in very low concentrations by analytical methods based on high-performance liquid chromatography (HPLC) and amperometric detection. Because reproterol contains two phenolic hydroxy groups, it seemed worthwhile to try amperometric detection, especially because ultraviolet and fluorescence detection of underivatized reproterol does not offer sufficient sensitivity.

By using HPLC, some advantages over GC may be obtained with regard to stability, selectivity (both stationary and mobile phase can be altered) and sensitivity, as relatively large amounts of sample can be injected without overloading the column. The latter aspect opens the possibility to use on-line trace enrichment to purify the plasma samples and to concentrate the sample with regard to the reproterol concentration. Especially when compounds like reproterol need to be analyzed, which are difficult to extract from the biological matrix and which are present in very low concentrations, the on-line enrichment procedure may be of great advantage.

We now report a method based on on-line trace enrichment, reversed-phase HPLC and amperometric detection capable of detecting reproterol in plasma down to 0.5 ng/ml.

## EXPERIMENTAL

### *Chemicals*

Reproterol hydrochloride and the structural analogs, D4908 and D4959, used as internal standard (see Fig. 1) were a gift of Homburg/Degussa Pharma Gruppe, Frankfurt/M., G.F.R.

Disodium hydrogen phosphate dihydrate, tetrabutylammonium hydrogen sulphate ($TBAHSO_4$), propanol-1, perchloric acid 70% in water, citric acid and sodium hydroxide were of pro analysi quality and obtained from E. Merck (Darmstadt, G.F.R.).

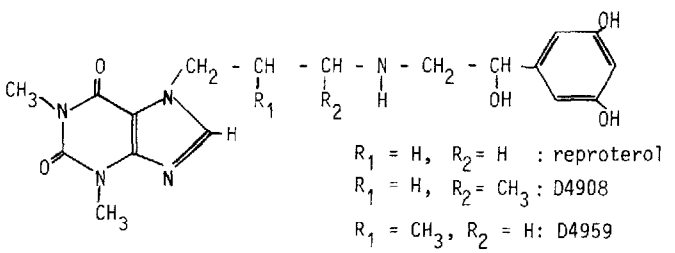


Fig. 1. The chemical structure of reproterol, D4908 and D4959.

Freshly glass-distilled deionized water was used for the preparation of all stock solutions of reproterol, D4908, D4959 and perchloric acid, as well as for the preparation of the mobile phases.

Carbon powder was purchased from Ringsdorff Werke (Ringsdorff Spektral-

kohle, RW-A höchster Reinheit; Bonn-Bad Godesberg, G.F.R.), high vacuum grease from Dow Corning (Seneffe, Belgium) and carbon paste (EA 267 c) from Metrohm (Herisau, Switzerland).

*On-line trace enrichment*

The set-up for the on-line trace enrichment procedure is schematically represented in Fig. 2. By injecting the sample in valve 1 ($V_1$) the loop L is filled. By switching $V_1$, the contents of loop L are pumped into $V_2$ on the concentration column CC. The wash mobile phase $S_1$ is made in such a way and the flush time $t_1$ chosen such that reproterol and D4908 are retained on the stationary phase present in CC whereas a great many accompanying compounds present in the sample are flushed away to waste. After a given time $t_1$, $V_2$ is switched and mobile phase $S_2$ is pumped through CC in the same direction as was $S_1$. Reproterol and D4908 are now flushed onto the analytical column AC and, after separation, enter the amperometric detector DC.

Deproteinized plasma samples can easily be handled this way.

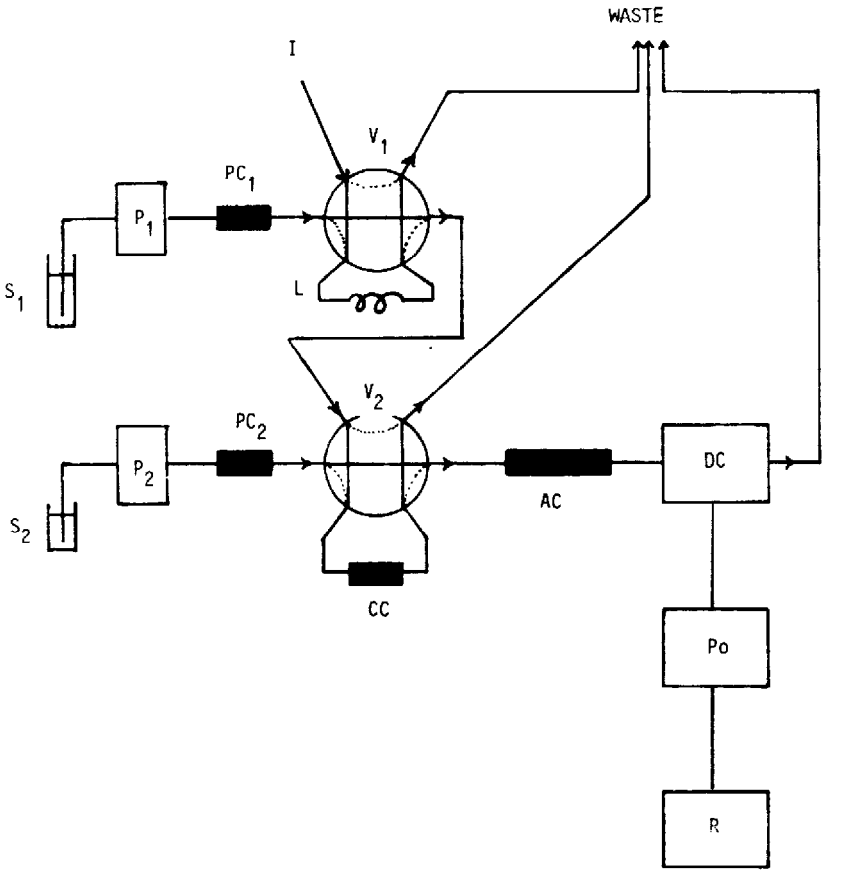


Fig. 2. A schematic representation of the set-up for the on-line trace enrichment procedure. $S_1$, $S_2$ = solvent reservoirs. $P_1$ = Model 740 pump (Spectra Physics, Eindhoven, The Netherlands). $P_2$ = Model 3500 B pump (Spectra Physics). $PC_1$, $PC_2$ = precolumns. I = injection. $V_1$, $V_2$ = 6-port valves (Model 7010, Rheodyne, Cotati, CA, U.S.A.). L = loop. CC = concentration column. AC = analytical column. DC = detector cell. Po = potentiostat. R = recorder BD40, 04/05 (Kipp & Zn, Delft, The Netherlands).

*Chromatography*

All columns were filled with Nucleosil 5 $C_{18}$, mean particle size 5 $\mu$m (Macherey, Nagel, Düren, G.F.R.).

The analytical column (150 × 4.6 mm I.D.) was slurry-packed according to a method described recently [10], which was modified slightly.

The concentration column CC was home-made, by filling a piece of stainless steel tubing (35 × 4.6 mm I.D.) with Nucleosil 5 $C_{18}$ to a height of 20 mm. The remaining part of the tubing is filled with a cylinder of tight-fitting PTFE (polytetrafluoroethylene) through which a capillary stainless steel (type 316) tubing with an external diameter of 1/16 in. is extended. On both sides of the Nucleosil column stainless steel frits are placed. The CC is depicted schematically in Fig. 3. This design was especially suited to be used in on-line trace enrichment of plasma samples with foreward flushing. The CC was packed by preparing a slurry as for the analytical column and this slurry was poured into the column under tapping with a rod.

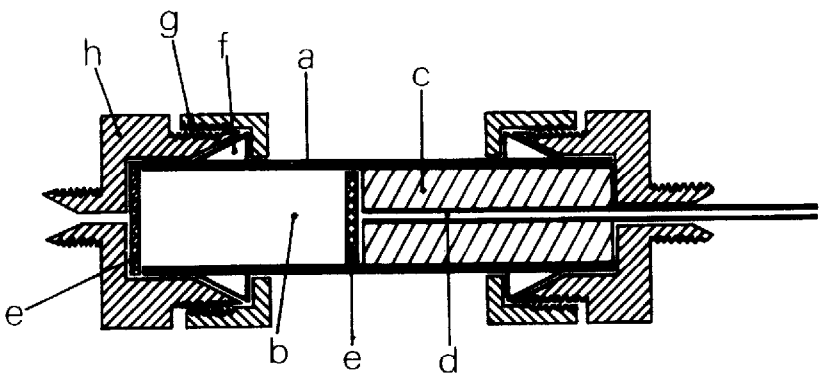


Fig. 3. A schematic representation of the concentration column. a = tubing. b = stationary phase. c = hollow cylinder. d = capillary tubing. e = frits. f = ferrules. g = nuts. h = snubber.

The precolumns were dry-packed with Nucleosil 5 $C_{18}$; they are taken up to provide on-line purification of the mobile phases. $PC_1$ and CC were cleaned each day by flushing with 15 ml of water—methanol (1:1). $PC_2$ and AC were flushed with 15 ml of water—methanol (2:1) each day.

The mobile phases were prepared from a mother buffer solution made by dissolving 7.12 g of disodium hydrogen phosphate dihydrate and 8.4 g of citric acid in 1970 ml of water and titrating this solution with 4 *N* sodium hydroxide solution to pH 7.0. This solution was diluted 1:1 with distilled water to give mobile phase $S_1$. Mobile phase $S_2$ was prepared by adding propanol-1 (to a concentration of 2.2–3.0%) and $TBAHSO_4$ (to a concentration of $4 \cdot 10^{-3}$–$6 \cdot 10^{-3}$ *F*) to the mother buffer (phosphate–citrate buffer, pH 7.0, 0.02 *F*).

The flow of $S_1$ was 1.0 ml/min and that of $S_2$ 1.2 ml/min.

The mobile phases were degassed by ultrasonication under vacuum and filtered through a cellulose acetate membrane filter (Schleicher and Schüll, Dassel, G.F.R., OE 67, 0.45 $\mu$m).

*Amperometric detection*

A home-made amperometric detector was used which has been described previously [11—13].

Some small modifications were made to improve the detector response. (1) The rotation speed was continuously adjustable as was the distance between working electrode and bottom of the cell. This improved the reproducibility of the detector at high oxidation potentials. (2) The diameter of the channel connecting the compartment containing the working electrode with that in which the reference electrode is present was enlarged to 5 mm. This lowered the internal resistance of the detector and increased the linear range.

The modified detector is depicted in Fig. 4. In the cell block (a) holes are drilled for the working electrode (b), the reference electrode (c) and the auxiliary electrode (d). Via the inlet (e) the eluent enters the cell and impinges on the working electrode, then streams along the carbon paste (f), through a channel (o) into a compartemt (p) containing the reference and auxiliary electrode and then to waste. The inlet consists of a stainless steel cylinder with a hole drilled in it (1.0 mm) which is screwed into the bottom of the working electrode compartment.

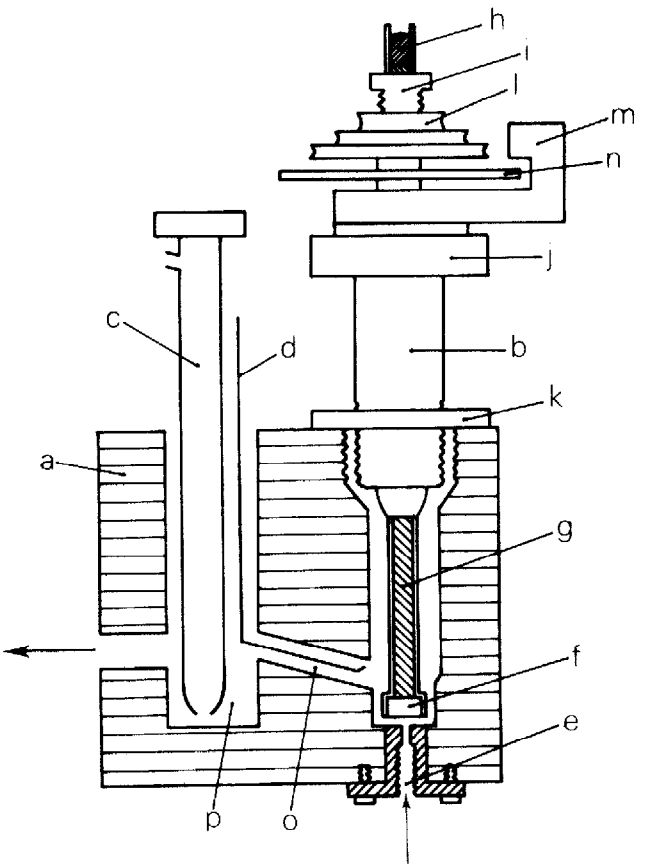


Fig. 4. Amperometric detector with rotating-disc working electrode, continuously adjustable nozzle height and rotation speed. a = cell block. b = working electrode holder. c = reference electrode. d = auxiliary electrode. e = inlet. f = carbon paste. g = brass rod. h = mercury. i, j, k = screws. l = pulleys. m = optical plate. n = plate with slieves in it.

The working electrode consists of a holder (h), which contains a piece of Kel-F tubing with a brass rod (g) in it to connect the carbon paste (g) to the mercury (h) on top of the rod in which the wire to the potentiostat is placed. The rod is placed in the holder with the screw (i). The holder is screwed into the cell block with screw (j) and fixed into place with screw (k). In this way the distance between the surface of the carbon paste and the surface of the metal bottom can be continuously and accurately adjusted. The rod is rotated in the holder with the aid of the pulleys (l) constructed on the top of the rod which are connected with an elastic string to a stepper motor. The rotation speed is measured by the optical cell (m) and the circle (n) screwed on the rod. With an external device the rotation speed is measured and the rotation velocity of the stepper motor regulated.

The electrode material consisted of carbon paste prepared as follows: carbon powder (0.66 g) and vacuum grease (0.33 g) were mixed in an agate mortar with a pestle until a homogeneous paste was obtained. Then Metrohm carbon paste (0.2 g) was pounded through the mixture in the same way. The auxiliary electrode was a platinum wire of diameter 0.6 mm. The oxidation potential was chosen to be 0.90 V vs. a Ag/AgCl—3 *M* KCl reference electrode.

The potentiostat was home-made and comparable to a LC-2A electronic controller (Bioanalytical Systems, Lafayette, IN, U.S.A.).

*Plasma sample preparation*

To investigate the properties of the method, spiked plasma samples were analyzed. In the experiments several lots of single donor human plasma and bovine plasma were used.

To 2.2 ml of plasma are added 50 $\mu$l of a stock solution of reproterol in water to give the concentrations desired. In some experiments also 50 $\mu$l of a freshly prepared stock solution of the internal standard were added. After 15 min equilibration time 1.8 ml of a solution of perchloric acid in water (6%) are added. After shaking on a Vortex mixer at maximum speed for three times 10 sec, the tubes are centrifuged at 0°C for 15 min at 2000 *g*. Then the supernatant is decanted into another tube and centrifuged for 5 min at 0°C at 4500 *g*. In this way a clear supernatant is obtained which is injected directly into $V_1$.

*Total procedure*

The total procedure can be taken together as follows: (1) A 2.2-ml volume of (spiked) plasma is deproteinized by adding 1.8 ml of a 6% perchloric acid solution, and shaking on a Vortex mixer at maximum speed for three times 10 sec. Then the tubes are centrifuged at 0°C for 15 min at 2000 *g*. The supernatant is decanted into another tube and centrifuged for 5 min at 0°C and 4500 *g*. (2) The loop L of the chromatographic system depicted in Fig. 2 is filled with 2.1 ml of the clear supernatant. Then the positions of the valves are changed as described in Table I. The exact time of injecting the next sample in loop L (in Scheme 1 marked by an asterisk) depends on the composition (origin) of the plasma sample. In some cases, late-eluting peaks were observed in the chromatograms and the moment of injection was postponed until the last compounds eluted from AC. Before this procedure was applied to the

TABLE I

TIME SCHEDULE FOR THE SWITCHING OF THE VALVES IN THE ON-LINE TRACE ENRICHMENT OF REPROTEROL-SPIKED PLASMA SAMPLES WHEN THE SET-UP DEPICTED IN FIG. 2 IS USED

| Time (min) | Position $V_1$ | Position $V_2$ | Function |
|---|---|---|---|
| | | | CC is equilibrated with $S_1$, AC and DC are stable |
| 0.00 | Load | Load | Injection sample in L |
| 2.00 | Inject | Load | Sample loaded onto CC |
| 10.00 | Inject | Inject | Reproterol is flushed onto AC; the HPLC separation (and the chromatogram) starts |
| 10.05 | Load | Inject | $V_1$ is cleaned |
| 14.00 | Load | Load | CC is equilibrated with $S_1$ |
| 17.00 | Load* | Load | L is filled with the next sample |
| 19.00 | Inject | Load | Sample loaded onto CC |

*Indicates injection of the next sample if no late-eluting peaks are observed.

samples, three aliquots of a spiked water sample (10—20 ng/ml) and a test plasma sample were injected.

A chromatographic run takes between 13 and 75 mins, and 3—6 plasma samples plus standards can be analyzed in duplicate within one day depending on the origin of the plasma sample.

The quantitative evaluation was done by measuring the peak height by hand.

## RESULTS AND DISCUSSION

### *Amperometric detection*

Reproterol could be oxidized at a carbon paste electrode in an aqueous medium; for example in the mobile phase used to elute reproterol from the concentration column, the drug was oxidized at potentials higher than 650 mV vs. an Ag/AgCl—3 *M* KCl reference electrode.

The choice of the potential used to oxidize reproterol depends on the reproducibility, selectivity and sensitivity needed. The reproducibility of the results is influenced by the amperometric detector itself. For example, if a potential is chosen at which no diffusion-limited current is obtained, if the applied voltage does not remain constant, if the electrode surface changes with time, or if poisoning of the electrode occurs. To investigate these factors, voltammograms were recorded on-line after an aqueous solution of reproterol or reproterol in plasma was led through the entire procedure. A current—voltage plot is given in Fig. 5.

Repetitive injection of the same aqueous solution at a constant potential of around 850 mV showed a decrease of the response in the first three injections; thereafter the response remained constant, indicating that the electrode surface reacted in a constant way. This behaviour is also observed in the amperometric detection of catecholamines [14]. Therefore all quantitative results mentioned in this article were obtained after three initial injections of an aqueous solution of reproterol.

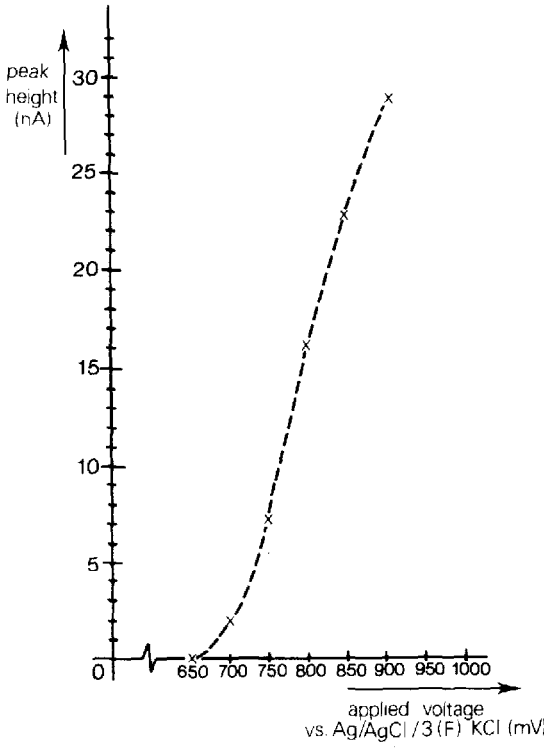


Fig. 5. Peak height (nA) measured at different potentials between working and reference electrode, when aliquots of an aqueous solution of reproterol (21 ng/ml, 3% perchloric acid) are injected in the set-up described in Fig. 2. $S_2$ consisted of phosphate–citrate buffer (pH 7.0, 0.01 *F*), propanol-1 (2.4%) and $TBAHSO_4$ ($5 \cdot 10^{-3}$ *F*). Nozzle height 1.0 mm. Rotation speed: 30 r.p.s.

In the voltammogram no flattening of the curve is observed.

At potentials higher than 950 mV, the residual current becomes too high, caused by the oxidation of water.

If the coefficient of variation (C.V.) is determined at 900 mV, by injecting five times a solution of reproterol in distilled water (2 ng/ml) a C.V. was found of 2.1% (i.e. including the on-line trace enrichment device).

The lower detection limit of reproterol in aqueous solution is 0.1–0.2 ng/ml and depends for the largest part on the amount of interfering compounds present in the sample, in the wash mobile phase or on the column, and not so much on high-frequency noise factors like electronic noise. This means that the signal-to-noise ratio (S/N ratio), which fixes the lower detection limit, may vary with the origin of the (plasma) sample. That is why no exact S/N ratios can be given. For plasma samples, the quantitation limit was usually found to be around 1.0 ng/ml. On the basis of the above observations, detection at a potential between 850 and 950 mV was routinely chosen, allowing quantitation of (sub)nanogram quantities of reproterol.

*Trace enrichment and chromatography*

Reproterol appeared to be very difficult to extract from plasma, with

recoveries below 40% using a variety of organic solvents. This is due to its rather polar character which results in a partition coefficient of 0.39 in the system *n*-octanol—0.02 *F* phosphate buffer, pH 7.4 [3].

Ion-pair extraction procedures with di-(2-ethylhexyl) phosphoric acid (DEHP) which appeared to be a suitable counter ion for resorcinol-type sympathicomimetics [6, 15] were also investigated. Though extraction from water could be performed, recoveries from plasma remained more than 40% lower.

By using on-line trace enrichment, several of the problems occurring with solvent extraction are circumvented. (1) The method is easy to apply: often only a deproteinization step is necessary for plasma. (2) The procedure is amenable to automation. (3) Possible losses due to adsorption (to glass during evaporation, for example) are minimized as well as losses which may occur during the transfer of small volumes. (4) By making an adequate choice of the stationary and mobile phases, a high degree of purification of the sample and a high selectivity can be obtained [16]. (5) Sensitivity can be increased by concentration effects (for example, by using a step gradient).

On-line trace enrichment has been used successfully for several years in the analysis of various compounds in different matrices and more recently also in the analysis of drugs in a complex matrix like biological fluids (serum [17, 18], plasma [18—23], urine [23]).

In our studies on trace enrichment, Nucleosil 5 $C_{18}$ appeared to be a suitable material to trap reproterol when an aqueous phosphate—citrate buffer (0.01 *F*, pH 7.0) is used as mobile phase.

When using this stationary phase for the enrichment procedure, the only plasma clean-up required is deproteinization, to prevent clogging of the concentration column. Satisfactory deproteinization was obtained by adding 1.8 ml of a solution of 6% perchloric acid [24] in distilled water to 2.2 ml of plasma. More than 100 injections of 2.1 ml of deproteinized plasma can be done on the same concentration column, without significant damage.

Several compositions of mobile phase to elute reproterol into the analytical column were tried for best performance. By using an elution mobile phase based on mixtures of phosphate—citrate buffer, sodium perchlorate and propanol-1, tailing peaks and bad efficiencies on various $C_{18}$ reversed-phase analytical columns resulted. (Altex Ultrasphere-ODS, 5 $\mu$m, 25.0 × 0.46 cm; $\mu$Bondapack $C_{18}$; Nucleosil 5 $C_{18}$, 15.0 × 0.46 cm; Hibar LiChrosorb RP-18, 10 $\mu$m, 25.0 × 0.40 cm).

When, instead of perchlorate, other ion-pairing agents were used like heptane sulphonate, high concentrations of organic modifier were necessary in order to obtain a reasonable retention time. This disturbs the performance of the carbon paste working electrode.

We found the most suitable mobile phase to be a solution of $TBAHSO_4$ in an aqueous phosphate—citrate buffer, containing a small amount of propanol-1, with which a highly efficient column behaviour was obtained, using Nucleosil 5 $C_{18}$ as stationary phase [25].

The retention time of reproterol in such a system can be governed in several ways. (1) By changing the pH of the buffer (and keeping the other constituents constant: propanol-1 5.0%, $TBAHSO_4$ $5 \times 10^{-3}$ *F*) from 6.5 to 8.0, the retention time of reproterol is increased from 4.6 to 8.4 min and the number of

effective plates decreased by 15%. (2) By changing the propanol-1 content in the mobile phase from 2.5 to 5.0% ($TBAHSO_4$ $5 \cdot 10^{-3}$ *F*) the retention time of reproterol changed from 12.8 to 5.0 min. (3) By increasing the $TBAHSO_4$ concentration from $5 \cdot 10^{-3}$ to $6 \cdot 10^{-3}$ *F* (2.6% propanol, $1 \cdot 10^{-2}$ *F* McIlvaine buffer pH 7.18), the retention time of reproterol decreases from 14.8 to 12.0 min. This would seem to be in contrast to the behaviour expected when ion-pair formation between reproterol and $TBAHSO_4$ is a major factor in the chromatographic behaviour of reproterol on a $C_{18}$ stationary phase. (4) The ionic strength also influences retenton time in the way that increasing ionic strength causes increasing retention times. For example, when the phosphate–citrate buffer (pH 7.0) is made 1.5 $\cdot 10^{-2}$ *F* instead of 1.0 $\cdot 10^{-2}$ *F* (propanol-1 2.8%; $TBAHSO_4$ $8.0 \cdot 10^{-3}$ *F*) a retention time of 8.5 min instead of 7.1 min is found.

With these parameters the retention of reproterol can be properly adjusted to the most suitable position in the chromatogram. We found that, for plasma samples, a mobile phase composition of phosphate–citrate buffer, pH 7.0–7.2, 0.01 *F*, propanol-1, 2.2–2.6%, and a $TBAHSO_4$ concentration of $5 \cdot 10^{-3}$ to $6 \cdot 10^{-3}$ *F* provided good chromatographic results coupled with an excellent clean-up and enrichment of the sample, as can be observed in Fig. 6.

The addition of $TBAHSO_4$ to the above mobile phase system decreased the retention time ($t_R$) of reproterol and diminished tailing of the peak.

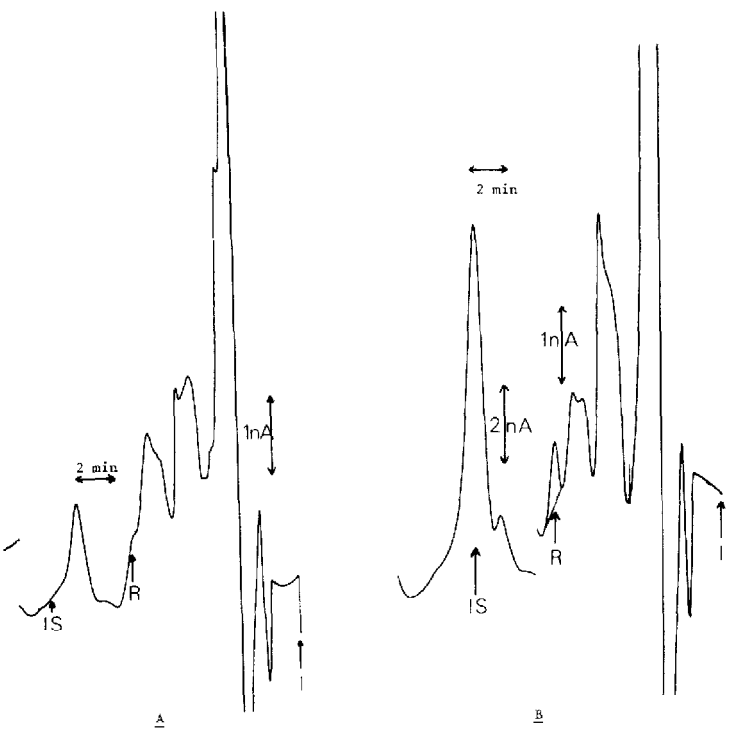


Fig. 6. Chromatograms of (A) a blank plasma sample and (B) a spiked plasma sample (0.55 ng reproterol HCl per ml of plasma). I represents the time of injection, R represents the reproterol peak and IS the peak of the internal standard D4908.

The choice for this elution mobile phase also has the advantages that reproterol is electrochemically very reactive in this medium and that the chance for isoquinoline formation (Pictet-Spengler reaction) is very small due to the absence of aldehydes or reactive aldehyde-forming agents [26].

With the latter chromatographic system, the properties of the analytical procedure were investigated. The enrichment method appeared to be dependent on two factors: (1) If the concentration column (CC) was in equilibrium with the wash eluent, before the next sample was injected. When 2.0 ml were used to equilibrate the CC, a 10% lower peak height was measured as compared to flushing with 16 ml of wash eluent. When 4.0 ml are used, CC approaches complete equilibrium. (2) The time the CC is flushed with the wash eluent, after the introduction of the sample. The peak height is inversely related to increasing volumes of $S_1$ used to wash the CC. When a complex sample like plasma is analyzed, a wash volume of 8 ml proved to be a good compromise.

It should be noticed that these relatively large flushing volumes put high demands upon the purity of the mobile phase $S_1$. The impurities present in $S_1$ will accumulate on top of the CC and (partly) be eluted when the CC is flushed with $S_2$. Thus, when the phosphate—citrate buffer is prepared, salts of highest quality must be used, together with freshly distilled water. Despite these precautions it appeared that impurities remained in $S_1$ and $S_2$ and that they originated from the salts (determined by making a gradient from 100% buffer to 100% methanol and measuring the eluent by ultraviolet detection at 254 nm). Therefore on-line purification of $S_1$ and $S_2$ was used.

*Internal standards*

D4908 and D4959 are closely related to reproterol and showed good chromatographic behaviour. However, we found that the two drugs did not improve the precision of the method. Coefficients of variation using peak height ratios of reproterol to internal standard were considerably larger than when using peak height of reproterol alone. This may be due to lower and less reproducible recoveries of the internal standards, which were found to be around 50%. Other factors may be differences in response at the electrode.

No further studies into these phenomena were undertaken nor did we investigate other potential candidates for internal standard. Instead, peak height of reproterol was measured and used as described below.

*Response, calibration, recovery and precision*

As mentioned above, when aqueous samples of reproterol were analyzed, the response decreased during the first three samples, and then remained constant during the rest of the day. Yet, when working with plasma samples we observed that after the first three aqueous samples the response continued to decrease slowly but steadily during the day. This may indicate that plasma contains compounds that influence the behaviour of reproterol in the system and/or the electrode surface.

In order to correct for this decrease in response and to obtain insight in the recoveries, a correction scheme as depicted in Fig. 7 was devised. The first three peaks represent the peak heights obtained when an aqueous solution of 5.0

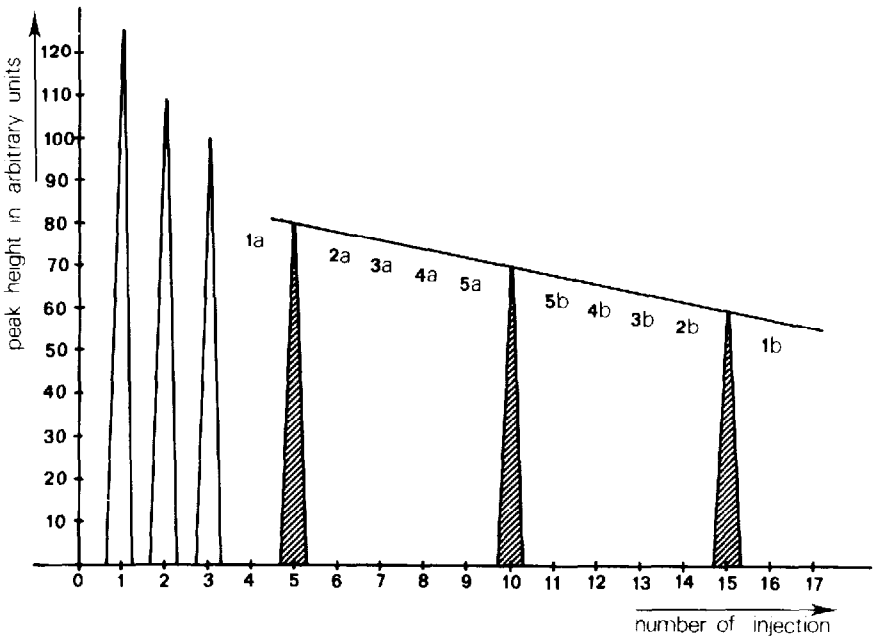


Fig. 7. A typical example of decrease of response when several plasma samples are injected.

ng/ml reproterol is injected. After the first three injections, the peak height for aqueous samples remains constant at $H_3$ (which represents 100% recovery). The shaded peaks at the numbers 5, 10 and 15 represent the peak heights obtained when spiked plasma samples (5.0 ng/ml) are injected during the day. From the latter the decrease in response can be calculated. The standards are made up in plasma from the same source as the plasma samples with the unknown concentration of reproterol. Taking into account the decrease in response, the relative recovery can be calculated by using $H_5$, $H_{10}$ and $H_{15}$ versus $H_3$. The injections numbered 1a—5a and 5b—1b represent plasma samples with an unknown quantity of reproterol. The "b" numbers are duplicates of the "a" numbers. Thus, by analyzing the plasma samples according to this scheme, the peak height can be corrected for the gradual decrease during the day. Of course the number of injections in the scheme can be enlarged by injecting more samples and standards.

When the above injection scheme was used for the analysis of plasma samples (spiked reproterol in the concentrations 0.55, 1.10, 1.84, 3.68 ng/ml), the linear calibration curve depicted in Fig. 8 was obtained. The mean values lie on a straight line of the equation $H_{repr.} = 1.42$ (conc. repr. in plasma) + 0.068, with a correlation coefficient of $r = 0.995$, indicating that the decay in response is accurately corrected for by the described procedures.

In Fig. 6 the chromatograms of blank plasma and of spiked plasma (0.55 ng/ml) are reproduced. These chromatograms, combined with the calibration curve, give an impression of the detection limit of the procedure. Because of the response changes during the day and between days, the lower limit of accurate determination is about 1 ng/ml in plasma.

The procedure is linear to at least concentrations up to 40 ng/ml. For the

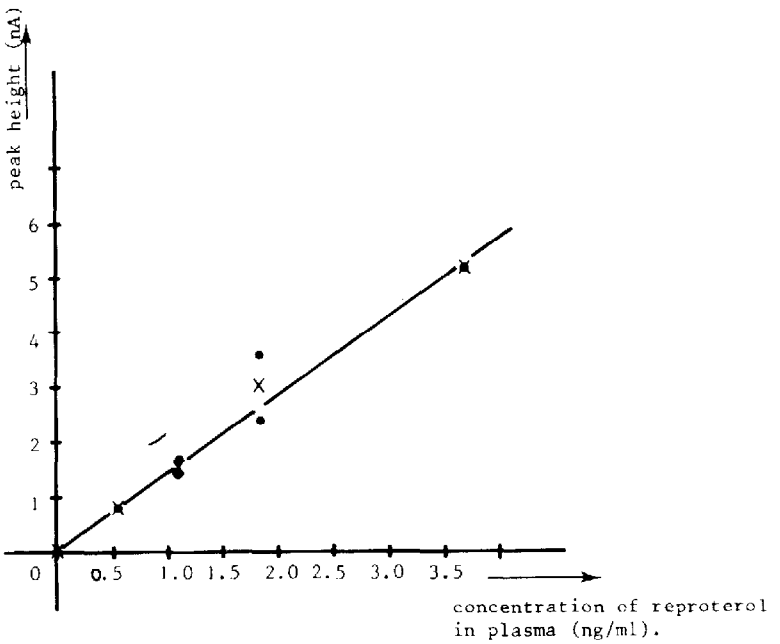


Fig. 8. Standard curve. Peak heights found when reproterol spiked plasma samples are analyzed according to the procedure described in the text. The crosses (x) represent the mean values of the two single determinations (•). No peak at $t_R$ = 8.3 min (= reproterol) was detectable in the blank plasma sample.

range 3—40 ng/ml a straight line was found with the equation $H_{repr.}$ = 1.01 (conc. repr. in plasma) — 0.74 with a correlation coefficient of $r$ = 0.998.

If the method of Haefelfinger and Wall [27] is used to combine the values of C.V. found for experiments at different concentrations on different days, a weighted mean C.V. of 7.8% was found for the concentration range 1—40 ng/ml. No significant difference in the value of C.V. was observed when plasma samples with a concentration of reproterol in the range 1—3 ng/ml or in the range 3—40 ng/ml, were analyzed.

Because of the decrease in response also no exact value of the recovery can be given. This is illustrated in Fig. 7. When the relative recovery (%) is defined as

$$\frac{\text{peak height reproterol in spiked plasma (ng/ml)} \times 100}{\text{peak height reproterol in spiked water (ng/ml) third injection}}$$

in our experiments recoveries were found in the range 60—90%. Since each plasma sample serves as its own recovery control (as indicated in Fig. 8) reliable quantitation is possible.

No significant differences were noted in recovery when plasma samples of different origin were analyzed.

It appeared that the plasma deproteinization procedure was a major factor in influencing the recovery. When 2.2 ml of blank plasma were spiked to a concentration of 3.0 ng/ml and deproteinized by adding 1.8 ml of a solution of 6% perchloric acid in distilled water and analyzed, a mean recovery of 72%

was found ($n$ = 3). When 2.2 ml of blank plasma were deproteinized in the same way and the supernatant was spiked, a mean recovery for the on-line trace enrichment device of 94% ($n$ = 3) was found. Higher concentrations of perchloric acid decreased recoveries, so as a compromise the 6% solution was used.

When plasma samples of unknown reproterol concentration have to be analyzed, blank plasma samples of the same person should be used as standards to correct for possible differences in recovery that may be present.

*Interferences*

We investigated the possible interference of some structurally related drugs in the quantitation of reproterol. It appeared that terbutaline, salbutamol, fenoterol, theophylline and the major metabolite of reproterol in humans (the isoquinoline derivative of reproterol [28] did not interfere.

*Applicability*

The applicability of the developed procedure is shown in Fig. 9, which depicts the plasma concentration—time curve when two tablets of Bronchospasmin® (containing 20 mg of reproterol HCl each) were given to a young healthy male volunteer. The tablets were swallowed together with 200 ml of water, and at certain times blood samples were collected in Vacutainer® tubes (with heparin). The tubes were stored in ice until centrifugation (4800 $g$, 0°C, 10 min). After centrifugation, two times 2.2 ml of the supernatant were brought to separate glass tubes (Sovirel® 15) and stored in the freezer (at

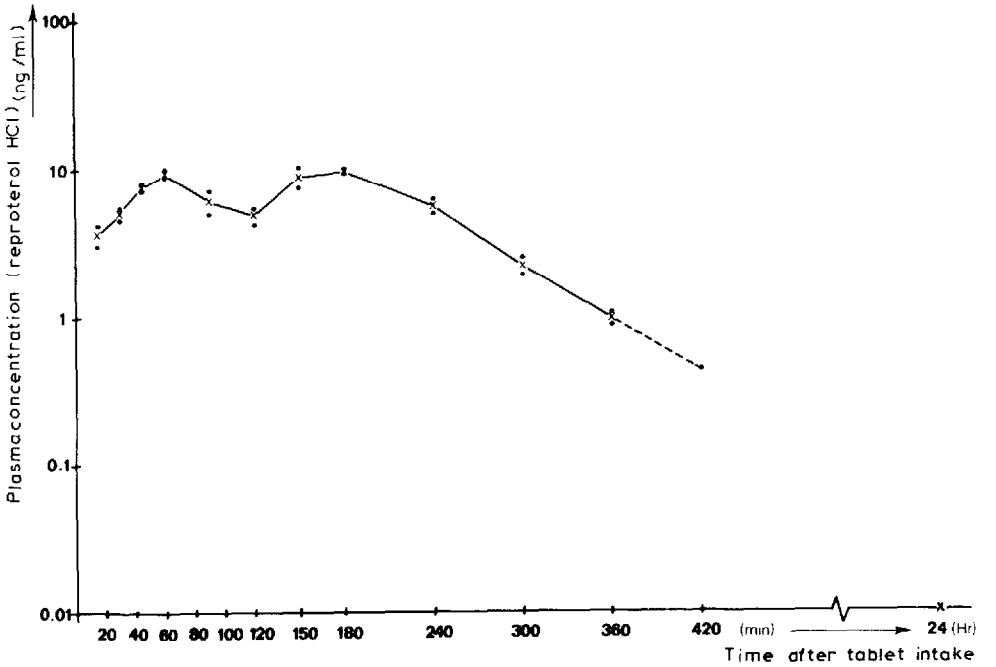


Fig. 9. Plasma concentration—time curve after oral administration of two tablets of Bronchospasmin® to a young healthy male volunteer. The crosses (x) represent the mean value of two determinations (•).

−20°C) until analysis. Details of the kinetic properties of reproterol in humans will be published elsewhere.

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