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Determination of fenoterol in human plasma by HPLC with fluorescence detection after derivatization

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Abstract

A new method for the determination of fenoterol is described, which uses HPLC separation with fluorescence detection. Dobutamine is employed as an internal standard. The separation was achieved on a short reversed phase column with a mobile phase consisting of water, acetonitrile and methanol. Prior to chromatography both analytes are derivatized with 9-chloroformyl-carbazole. Isolation of the analytes from plasma is carried out by liquid–liquid extraction into 2-butanol after protein precipitation with acetonitrile. The method is capable of estimating fenoterol concentrations in the sub-nanogram per ml range with sufficient accuracy and precision. The determination of fenoterol can now be carried out in the average laboratory without radiolabelled material.

Keywords: Fenoterol; HPLC assay; Derivatization

1. Introduction

Fenoterol is an adrenoceptor agonist with predominant ß$_2$-adrenoceptor activity [1,2]. The compound has been used for tocolysis and in the treatment of asthma for more than two decades. Recently it has been shown that fenoterol significantly increases the production of the glycoprotein hormone erythropoietin (EPO) in an experimental setting as well as during tocolysis [3,4].

During the drug development phase fenoterol concentrations in samples from clinical studies have been assayed by a highly sensitive (0.02 ng/ml) radioimmunological assay [5]. This assay method is not available any longer, since the production of radiolabel has been abandoned. Other methods for the determination of fenoterol

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have been described in the literature including GC-MS techniques [6], absorptive voltammetry [7] and enzyme immunoassay [8]. One HPLC method can also be found, which has been used for analyses in pharmaceutical preparations [9].

We report here a new method for the determination of fenoterol in human plasma which uses fluorescence detection after suitable derivatization of the analyte. The method was developed for use in the context of a clinical study of the effect of fenoterol on the EPO production under long-term tocolysis.

2. Materials and methods

2.1. Apparatus

The chromatographic system consisted of a Merck–Hitachi model 655A-12 pump with a L-5000 controller, a Merck–Hitachi model 655A-40 autosampler, and a Merck–Hitachi model F1000 fluorescence detector (Merck, Darmstadt, Germany). The detector was operated at an excitation wavelength of 285 nm and emitted fluorescence was measured at 345 nm. The detector signal was processed by a D2000 chromato integrator (Merck) or alternatively registered by the software package CSW ANDROMEDA (Techlab, Erkerode, Germany) on a personal computer. For the separation a LiChrospher 60 RP-Select B column (125 mm × 4 mm, 5 μm particle size, Merck) was employed. The mobile phase consisted of acetonitrile, methanol and water (solvent A: 72/16/12 (v/v/v); solvent B: 66/16/18 (v/v/v)). A gradient program was run from the controller starting with 100% solvent B for 10 min and changing to 100% solvent A between 10 and 10.1 min. After 19 min the system was switched back to solvent B for another 4 min. The flow rate was kept at 1.0 ml/min at ambient temperature, which resulted in a back-pressure of typically 90 bar. The integrator was started with a time delay of 10 min to exclude the broad front peak from the chromatogram. Therefore, 10 min had to be added to all times printed on chromatograms to yield total retention times.

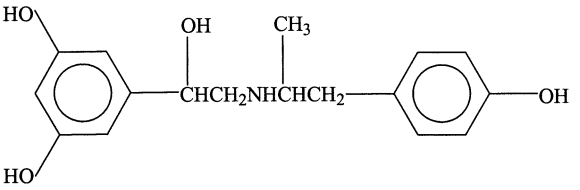

Fig. 1. Fenoterol.

2.2. Reagents

Fenoterol hydrobromide, 50 μg/ml in water was obtained from Boehringer-Ingelheim, (Biberach, Germany). Dobutamine hydrochloride as the internal standard was from Eli Lilly (Indianapolis, IN). Sodium hydroxide, boric acid, phosphoric acid, di-sodium hydrogen phosphate, methanol, acetonitrile, 2-butanol and hexane were from Merck. 9-Chloroformyl-carbazole (CARB) was purchased from Fluka (Deisenhofen, Germany). All reagents were used as obtained without further purification. Doubly distilled water was used in all experiments.

2.3. Sample preparation

Samples were kept at −20 °C prior to analysis. After thawing, samples were briefly centrifuged to remove debris. In a glass centrifuge tube with ground glass stopper 1 ml of sample was then spiked with 10 ng internal standard (10 μl of a 1 μg/ml aqueous solution) and then mixed with 2 ml acetonitrile on a reciprocating shaker for 10 min. After centrifugation at 4000 rpm for 5 min the supernatant (2.5 ml) was transferred to a second series of glass tubes. The solution was consequently dried under a nitrogen stream at 80 °C (≈40 min). The residue was then reconstituted in 1 ml phosphate buffer (0.1 M, pH 6.0) and as above extracted for 10 min into 2 ml 2-butanol. After centrifugation (5 min, 4000 rpm) the supernatant was transferred into a tapered centrifuge tube and again dried under nitrogen at 80 °C (≈30 min). The residue was this time dissolved in 40 μl borate buffer (0.2 M, pH 9.0) and then 110 μl acetonitrile were added. The sample was now derivatized with 20 μl carbazole solution

(2.66 mg/ml in acetonitrile) for 60 min at room temperature.

After completion of the derivatization reaction the sample was transferred into a polypropylene cup (Eppendorf type). The derivatization vessel was rinsed with 300 μl hexane and the hexane added to the sample. The resulting solution was briefly vortexed and then centrifuged for 2 min at 15 000 rpm. Of the upper hexane layer 200 μl were discarded and the wash-step was repeated with further 300 μl of hexane.

Finally 100 μl of the lower layer were removed into micro injection vials. Typically, 60 μl were chromatographed.

2.4. Clinical study

Samples were collected from 24 pregnant women who underwent long-term tocolysis with fenoterol (80 μg/h) for medical reasons and were healthy otherwise. Up to four blood samples per patient were taken at 2, 12, 96 and 144 h after the start of the fenoterol treatment. Written informed consent was obtained from each participant and the study protocol was approved by the local ethics committee. The clinical study will be reported in full detail elsewhere [10].

2.5. Calculations and calibration

Stock solutions were prepared containing 50 and 5 ng/ml fenoterol hydrobromide in water, respectively. These solutions were used for four weeks and then discarded. The stock solution of the internal standard was renewed once a week. Calibration samples were prepared in bovine serum containing 0.1, 0.25, 0.5, 1.0 and 2.5 ng/ml fenoterol as the hydrobromide. A complete set of calibration samples and an additional reagent blank was analysed daily together with the unknowns.

Peak area ratios (fenoterol/dobutamine) were employed for the calculation of calibration functions by least-square linear regression. Assuming a heteroscedastic error distribution, the inversed squared concentrations were used as weights. Concentration results were converted to nmol/l fenoterol by multiplication with 2.602. Fenoterol peaks in chromatograms, which corresponded to less than 0.08 ng/ml (80% of the lowest calibrator) were considered not quantifiable (Fig. 1).

Recoveries were calculated by comparing peak areas of samples after the work-up procedure with areas obtained from aliquots of the stock solutions which were directly derivatized and chromatographed.

2.6. Quality controls

Two quality control samples containing 0.25 and 1.0 ng/ml fenoterol hydrobromide, respectively, were included in each series of samples analysed.

3. Results

Under the conditions described in Section 2 a good separation of fenoterol, the internal standard and remaining endogenous material was achieved (Fig. 2A and B). Retention times for fenoterol varied between 15.4 and 16.7 min, while dobutamine was eluted between 17.9 and 19.5 min after injection. The work-up procedure yielded sufficiently clean extracts with recoveries that allowed quantitation of fenoterol concentrations down to 0.1 ng/ml (Fig. 2). In 92 patient samples fenoterol concentrations between 0.08 and 3.61 ng/ml (corresponding to 0.208 and 9.393 nmol/l, respectively), were found. Fig. 3 shows chromatograms of two samples of a typical patient receiving 0.08 mg/h fenoterol hydrobromide. The specimen taken 2 h after the start of the infusion showed a concentration of 0.69 ng/ml (equivalent to 1.795 nmol/l), the other sample was collected after 12 h and contained 1.91 ng/ml (4.97 nmol/l).

Recoveries for fenoterol decreased in an apparently concentration dependent fashion with increasing concentrations from 95% at 0.1 ng/ml to 42% at 2.5 ng/ml. Dobutamine recoveries were considerably lower with 18% at the 10 ng/ml level.

A linear calibration function could be established over the concentration range with an average slope of 0.2133 (95% CI 0.20776–0.21884) and an intercept of 0.0421 (95% CI 0.03544–0.04876).

The intraday variability ranged from 10% at the 0.1 ng/ml level to 13% at the 2.5 ng/ml level. The interday variability was 33% at the low end of the

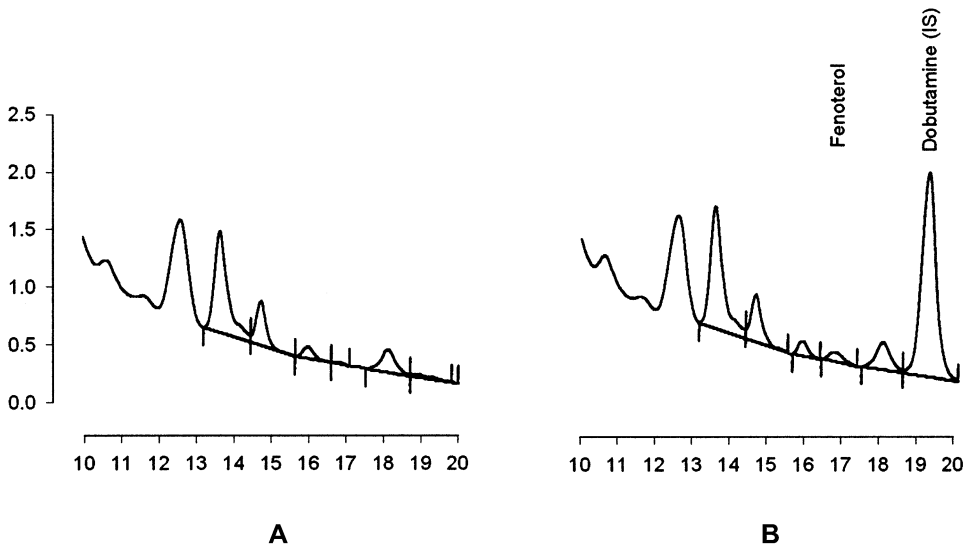

Fig. 2. Representative chromatograms of (A) a plasma blank and (B) a 0.1 ng/ml calibration sample. (X-axis retention time in min, Y-axis signal in mV) Peaks were for fenoterol at 16.68 min after integrator start and for dobutamine at 19.13 min.

calibration range and 22% at the high end ($n = 16$).

For the quality control samples spiked with 0.25 ng/ml fenoterol on average 0.21 ng/ml (s.d.: 0.08 ng/ml, $n = 27$) were calculated while the samples containing 1.0 ng/ml fenoterol yielded a mean of 0.90 ng/ml (s.d.: 0.25 ng/ml, $n = 29$).

4. Discussion

Fenoterol is a low protein bound, high extraction drug with a elimination half-life of 7 h [1,11]. With dose rates of 30–120 μg/h fenoterol plasma concentrations in the range from 0.2 to 1 ng/ml are to be expected [12]. The required sensitivity could be achieved with the aid of a derivatization step. However, there was a price to be paid for this achievement. Due to the fact that derivatization reactions seldom proceed selectively a number of by-products was generated which needed to be removed. Thus, the resulting sample clean-up was more complex than the average method. The method described in this work is an improved and validated version of the procedure first lined out in the dissertation by S. Kramer [13].

The chromatographic separation of fenoterol and the internal standard dobutamine was achieved with acceptable retention times ($k' < 10$) and good peak shape. The eluting power of the solvent used at the beginning of the cycle (solvent B) is very weak for the analytes. However, a broad front peak was eluted and thus safely removed. Therefore, the change of solvent composition during chromatography is more of a final washing followed by isocratic elution step than a true gradient separation. Solvent A was used for the elution of the analytes from 10 min after injection onwards when the output signal had dropped to approximately 20% full scale (Fig. 2). Minute adjustment of the solvent composition using a few percent more methanol instead of acetonitrile were occasionally necessary to optimize the separation of the analytes from co-eluting material. A throughput of approximately three samples per h can be maintained with an autosampler.

Isolation of the analytes from the matrix could be obtained with sufficient yield using 2-butanol. While 82% of fenoterol were recovered, only 33% of dobutamine were extracted. This is the main reason for the differences in the overall recoveries between compounds. However, 2-butanol cannot

be used to extract compounds of interest from plasma directly due to a lack in phase separation. Consequently the protein precipitation step was introduced. The pivotal derivatization step requires a solvent with a high organic content (around 50% acetonitrile) and a large excess of reagent even with pure stock solutions of analytes. While multiple derivatization can take place with fenoterol, which has five groups potentially reactive with CARB, only one fenoterol related peak was observed in the chromatograms. The same holds true for dobutamine. This indicates that the reaction proceeds in a reproducible and uniform way. The addition of hexane after completion of the derivatization intended to remove non-polar material, e.g. excess reagent, and improved the transfer of the sample prior to the final centrifugation step.

The use of plastic tubes and stoppers during the sample extraction caused the presence of artificial peaks in the chromatograms which interfered with the separation. It is suspected that this effect was due to plasticizers.

Reproducibility and accuracy as judged on the basis of the QC result are satisfactory. The slight bias, which was observed in the calculated means could possibly be due to the aging of the control samples. However, a clear trend could not be observed.

The chromatographic system employed does not separate the enantiomers of fenoterol. For this purpose either a chiral stationary phase is required, or a chiral ion pair reagent in the mobile phase or a derivatization step including an optically active compound. Preliminary results indicate [13], that an enantio-selective separation of the fluorescing derivatives of fenoterol and dobutamine can be achieved on a Chiracel OJ-R column. It remains to be evaluated how the assay sensitivity would be affected under such conditions. None of the methods reported in the literature offers separation of the enantiomers of fenoterol, even though the stereoselective formation of fenoterol glucuronides has been investigated.

The method reported in this work is well suited for studies which generate a moderate sample-load. While it does not have the full sensitivity of the radioimmunological assay, it can be used with equipment, which is today found anywhere and with off-the-shelf reagents.

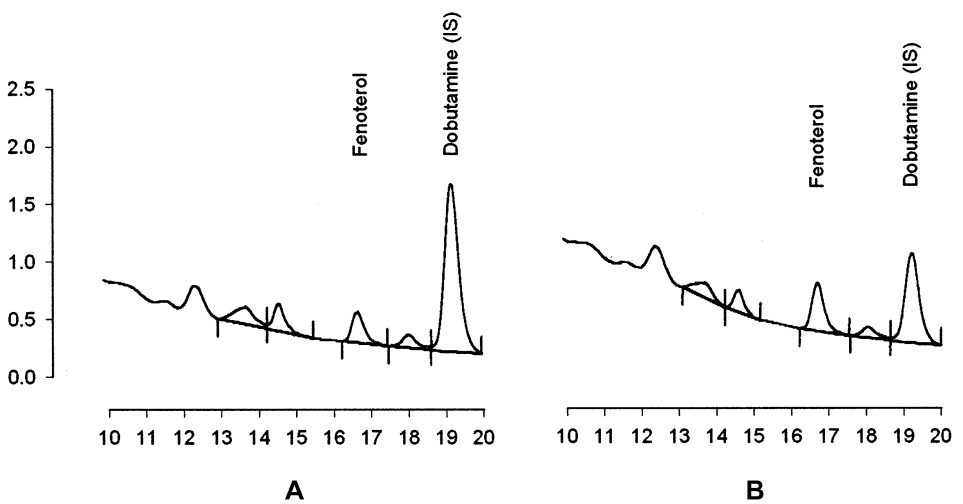

Fig. 3. Fenoterol concentrations in patients under long-term infusion. (X-axis retention time in min, Y-axis signal in mV). Samples were taken from subject no. 14 at (A) 2 h and (B) 12 h . Peaks in (A) were at 16.68 min for fenoterol and at 19.16 min for the IS. The fenoterol peak corresponds to 0.69 ng/ml. Peaks in (B) were at 16.69 min for fenoterol and at 19.17 min for the IS. The fenoterol peak represents 1.91 ng/ml.

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