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High-performance liquid chromatographic determination of the β_2-selective adrenergic agonist fenoterol in human plasma after fluorescence derivatization

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Abstract

A sensitive high-performance liquid chromatographic method has been developed for the determination of the β_2-selective adrenergic agonist fenoterol in human plasma. To improve the sensitivity of the method, fenoterol was derivatized with *N*-(chloroformyl)-carbazole prior to HPLC analysis yielding highly fluorescent derivatives. The assay involves protein precipitation with acetonitrile, liquid–liquid-extraction of fenoterol from plasma with isobutanol under alkaline conditions followed by derivatization with *N*-(chloroformyl)-carbazole. Reversed-phase liquid chromatographic determination of the fenoterol derivative was performed using a column-switching system consisting of a LiChrospher® 100 RP 18 and a LiChrospher® RP-Select B column with acetonitrile, methanol and water as mobile phase. The limit of quantitation in human plasma was 376 pg fenoterol/ml. The method was successfully applied for the assay of fenoterol in patient plasma.

Keywords: Fenoterol

1. Introduction

Fenoterol, 1-(3,5-dihydroxyphenyl)-2-{[1-(4-hydroxyphenyl)-2-propyl]amino}-ethanol (Fig. 1), is a β_2-selective adrenergic agonist which is routinely used as a tocolytic agent [1]. After oral administration fenoterol plasma levels are often too low to obtain tocolysis. Thus, for a tocolytic therapy the parental administration mode is preferred [2,3]. Despite the widespread use of fenoterol for tocolysis for more than two decades, very little is known about the relationship between fenoterol plasma concentration, pharmacological effect and side effects [4]. This is due to the very low plasma concentrations ranging from 600 to 2000 pg/ml after continuous i.v.-infusion of 0.5–3 μg/min [5]. Fort the determination of these low fenoterol plasma levels a radioimmunoassay has been described [6]. However, the antibodies are difficult to prepare and the assay is

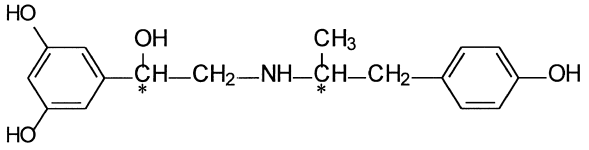

Fig. 1. Fenoterol.

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not commercially available. The aim of the present work was to develop a highly sensitive HPLC method for the determination of fenoterol.

2. Experimental

2.1. Chemicals and reagents

Fenoterol hydrobromide was kindly donated by the Boehringer Ingelheim KG (Germany). The internal standard dobutamine was obtained from ratiopharm (Germany) as dobutamine ratiopharm® injection powder. *N*-(chloroformyl)-carbazole (CARB) was purchased from Fluka (Germany). The solvents used for HPLC were of HPLC grade and obtained from BAKER (Netherlands). Isobutanol and *n*-pentane were of analytical grade and purchased from Baker. All other reagents and chemicals were of analytical grade.

2.2. Solutions of standard and derivatization reagent

Standard solutions of fenoterol hydrobromide and *N*-(chloroformyl)-carbazole were prepared freshly every day. The borate buffers were prepared once a week and stored at −4°C. Fenoterol hydrobromide standard solutions were prepared by dissolving 1 mg of fenoterol hydrobromide in 100 ml water. To obtain the final concentration, the solution was diluted with acetonitrile. *N*-(chloroformyl)-carbazole as well as dobutamine were dissolved in acetonitrile.

2.3. Chromatography

The HPLC system consisted of a Merck-Hitachi 6200 intelligent pump, a Merck-Hitachi F-1050 fluorescence detector and a Merck-Hitachi D-2000 integrator (Merck, Germany). The autosampler MSI-T-660 was obtained from Kontron (Kontron, Germany). A 50-μl injection valve was used. The excitation wavelength of the fluorescence detector was set at 287 nm, the emission wavelength at 345 nm. The columns used (LiChrospher® 100 RP 18 and LiChrospher® RP-Select B column 250×4.6 mm, 5 μm) were obtained from Merck (Germany).

2.4. Human samples

All samples (human blank plasma and patient plasma) were stored at −20°C until analysis. Patient plasma samples were collected in the Department of Clinical Pharmacology Göttingen (Universitätsklinik Göttingen, Klinische Pharmakologie, Prof. Dr. H. Gleiter). All patients received a continuous i.v.-infusion of fenoterol for treatment of preterm labour. The dosage and duration of therapy were determined by therapeutic goals.

2.5. Derivatization procedure of standard solutions

50 μl of fenoterol standard solution, 60 μl of internal standard solution (99.5 ng/ml), 40 μl of borate buffer (200 mmol/l, pH 9.0) and 20 μl of CARB solution were vortexed and treated in the same way as described for plasma samples.

2.6. Extraction of plasma samples

To 900 μl of human plasma, 50 μl fenoterol standard solution was added. The samples were briefly vortexed. After addition of 2 ml of acetonitrile the samples were vortexed, ultrasonicated (1 min), shaken (5 min) and centrifuged (5 min, 4000 rpm). The supernatant was removed and evaporated to dryness at 65°C under a gentle stream of nitrogen. The residue was dissolved in 1 ml of borate buffer (100 mmol/l, pH 9.0). Internal standard solution (60 μl) and 2 ml of isobutanol were added. After vortexing (30 s), ultrasonicating (1 min), shaking (10 min) and centrifugation (10 min, 4000 rpm) the upper layer was removed and evaporated to dryness under a gentle stream of nitrogen.

2.7. Derivatization procedure of plasma samples

Acetonitrile (110 μl), 40 μl of the borate buffer and 20 μl of the CARB solution were added to the dry residue. The samples were vortexed (30 s), ultrasonicated (2 min) and vortexed again for 20 s. After a reaction time of 60 min, 500 μl of *n*-pentane were added and the reaction mixture was vortexed for 30 s. After centrifugation (1 min, 4000 rpm) the upper layer was discarded and the extraction with *n*-pentane was repeated twice. After 10 min, 70 μl of

acetonitrile were added and the reaction mixture was ready for injection into the chromatographic system.

2.8. Chromatographic determination of total fenoterol

The separation was performed on a LiChrospher®60 RP Select B column followed by a LiChrospher® 100 RP 18 column using a column switching system. The mobile phase consisted of acetonitrile–methanol–water (standard solution: 81/13/6, v/v/v, plasma samples: 80/13/7, v/v/v). After injection of the sample, the flow was set at 1.0 ml/min and changed to 1.5 ml/min after 7 min (standard solution) or 1.7 ml/min after 7 min (plasma samples). Column switching was done after 6.3 min. The retention times found were 38.9 min for fenoterol and 52.70 min for the internal standard.

2.9. Recovery after extraction from human plasma and derivatization

The rate of the recovery was 75% from spiked plasma samples. Two different concentrations were investigated. The rel. deviation was 10.76 and 5.69%, respectively.

2.10. Linearity

The calibration curve was linear over the range evaluated (300–1600 pg/ml) with a correlation coefficient of 0.992 for the derivatization of fenoterol standard solutions and 0.990 after extraction and derivatization of plasma samples.

2.11. Stability of the derivatives

Fenoterol standard solution (50 μl, 126 μg/ml water), 20 μl of the borate buffer, 20 μl of the *N*-(chloroformyl)-carbazole solution and 80 μl acetonitrile were vortexed. After 1 h the mixture was diluted with 1 ml acetonitrile. Aliquots were stored at ambient temperature or at −20°C until analysis.

2.12. Accuracy and precision

For calibration, plasma samples containing 338, 676 and 1351 pg fenoterol were prepared by adding fenoterol standard solutions to blank plasma. Precipitation, extraction, derivatization and chromatographic separation were performed as described above. Each concentration was prepared three times. For determination of accuracy and precision, two different concentrations were determined on three different days. Each concentration was assayed six times.

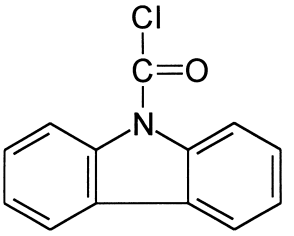

Fig. 2. *N*-(Chloroformyl)-carbazole.

3. Results and discussion

CARB (Fig. 2) was originally developed as reagent for the derivatization of amino acids [7,8]. The present work demonstrates that CARB is also an excellent label for drugs like fenoterol and dobutamine (Fig. 3), the latter being used as internal standard.

3.1. Derivatization procedure and chromatographic separation

The derivatization of picomolar amounts of fenoterol required a large excess of CARB reagent. The separation of excess of CARB, matrix compounds, fenoterol, derivative and dobutamine derivative was successful only after a cleaning procedure. To remove interfering peaks originating from the CARB-excess, the derivatized solutions were extracted with *n*-pentane which was discarded.

For separating one remaining interfering peak, a column-switching system was used consisting of two reversed-phase columns.

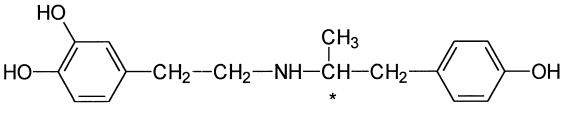

Fig. 3. Dobutamine.

Fenoterol shows five functional groups which may be derivatized with CARB. The reaction of the aliphatic hydroxyl function with CARB is unlikely [7]. Dependent on the pH of the reaction mixture, the secondary amino function and/or the phenolic hydroxyl functions are likely to be labelled with CARB. Using borate buffer of pH 9.0, a four-fold derivatized was the main product obtained. The proposed structure of this derivative was supported by LC–MS analysis. The mass signals of 1-, 2-, 3- and 4-fold derivatized fenoterol were found. Further fragmentation confirmed the derivatization of the phenolic hydroxyl functions [8].

Dobutamine proved to be a suitable internal standard, eluting later than the fenoterol derivative. Like fenoterol, dobutamine contains three phenolic hydroxyl functions in addition to the secondary amino function. Thus dobutamine may also form a four-fold labelled derivative. No interfering peaks occurred after HPLC analysis. The separation of the fenoterol and dobutamine derivatives after derivatization of stock solutions is shown in Fig. 4.

A reaction time of 40 min was sufficient to obtain highly fluorescent derivatives. Extension of the reaction time did not increase the yield.

To elute the labelled fenoterol and dobutamine derivative from the reversed-phase columns, an unpolar mobile phase was required consisting of acetonitrile, methanol and water (80/13/7, v/v/v). Under these conditions, the fenoterol derivative was eluted with very long elution times after 38.9 min and the derivative of the internal standard after 52.7 min. When a higher concentration of acetonitrile or methanol was used in order to shorten the time of analysis, the matrix compounds coeluted. The separation of the fenoterol and dobutamine derivative after extraction from spiked plasma and following derivatization is shown in Fig. 5.

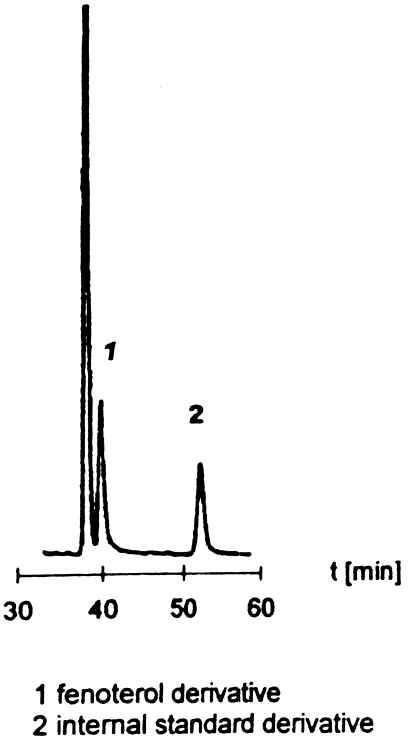

Fig. 4. Achiral separation of the fenoterol (500 pg/ml) and the internal standard derivative after derivatization of stock solutions.

3.2. Validation of the method

Linearity was studied before and after extraction of fenoterol from plasma (Tables 1 and 2). After extraction of fenoterol from plasma and derivatization with CARB, the method was linear for concentrations ranging from 338 to 1688 pg/900 μl. The correlation coefficient r was 0.992 (standard solution) and 0.990 (spiked human plasma), respectively.

The within-day precision and accuracy of the assay are shown in Table 3. Limits of a 20% deviation were accepted. The quantitation limit was found to be 376 pg/ml plasma or 100 pg on column. Thus, the assay is adequate to measure low fenoterol concentrations after a continuous i.v.-fenoterol therapy.

The recovery after extraction of fenoterol from human plasma and following derivatization with CARB at a high and as well at a low concentration level was found to be 75% (Table 4).

The fenoterol derivatives were stable over a period of 24 h when stored at ambient temperature. After storage at −20°C, almost no degradation of the fenoterol derivatives was observed after 13 days (Table 5).

Fenoterol is easily oxidised by exposure to air. This reaction is especially likely to proceed at

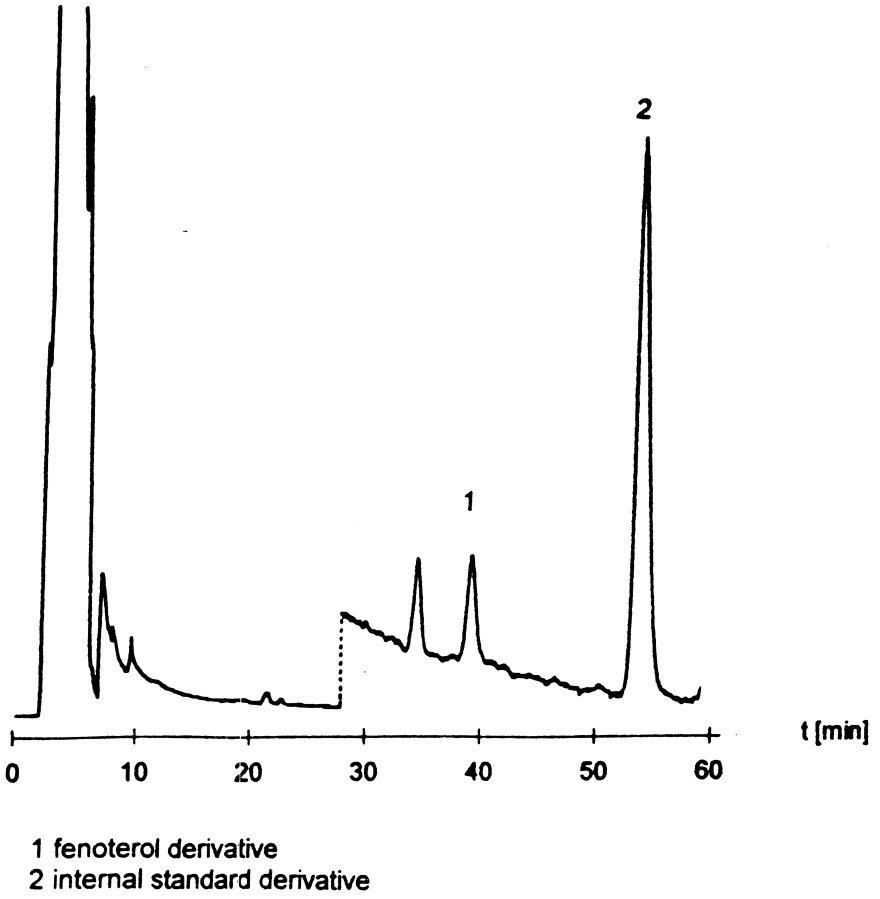

Fig. 5. Achiral separation of the fenoterol and dobutamine derivative after extraction of fenoterol (550 pg/ml) and the internal standard from plasma and following derivatization.

Table 1
Linearity of the assay before extraction[a]

Conc. (pg/170 μl)	Avg. area (fenoterol/dobutamine)	SD	SD_{rel} (%)
326	0.1761	0.0016	0.90
652	0.3247	0.0256	7.85
978	0.4539	0.0213	4.69
1304	0.5225	0.0450	8.62
1630	0.6620	0.0346	5.22

[a] Parameter of linear regression: $f(x)=0.000356x+0.0812$, $r=0.992$.

Table 2
Linearity of the assay after extraction[a]

Conc. (pg/900 μl)	Avg. height (fenoterol/dobutamine)	SD	SD_{rel} (%)
338	0.5347	0.0797	14.91
675	0.6541	0.1281	19.59
1013	0.8526	0.0277	3.24
1351	0.9100	0.0883	9.70
1688	1.0670	0.0496	4.65

[a] Parameter of linear regression: $f(x)=0.000391x+0.4074$, $r=0.990$.

alkaline pH. The best recovery from plasma using organic solvents can only be achieved after extraction from alkalized samples. Thus, spiked sam-

Table 3
Within-day precision and accuracy

Concentration calculated	Concentration found (n=6)	C.V. (%)
Day 1		
338	343	14.53
1351	1334	9.84
Day 2		
338	317	13.20
1351	1229	7.96
Day 3		
338	336	10.21
1351	1483	13.39

ples which were prepared for the calibration had to be prepared simultaneously with the samples for the assay.

3.3. Applications

The method was used to measure fenoterol concentrations in patient plasma. Fenoterol plasma levels of three patients receiving a continuous i.v.-infusion of 2 μg fenoterol/min which were determined to be between 0.30 and 1.50 ng/ml are shown in Fig. 6.

4. Conclusion

The sensitive HPLC assay described above is suitable to determine the very low fenoterol concentrations in patient plasma after a continuous i.v.-infusion of 2 μg fenoterol/min. High sensitivity was obtained by labelling fenoterol with *N*-(chloroformyl)-carbazole and following fluorescence detection. The assay was successfully used for the determination of fenoterol in patient plasma.

Fenoterol is chiral and is used as a racemate. The enantiomers could be separated by chiral HPLC resolution on different chiral stationary phases or after derivatization with optically active reagents [8]. However, the sensitivity of this chiral assay was not sufficient in order to determine the extremely low concentrations of fenoterol present in human plasma.

Table 4
Recovery

Conc. (pg/50 μl)	Avg. area plasma	Avg. area standard	SD_{rel} % plasma (n=6)	SD_{rel} % standard (n=3)
472	0.1984	0.2857	10.76	10.76
1422	0.7326	1.0139	15.87	5.69

Table 5
Stability of the fenoterol derivatives

Storage time	Avg. height (n=3)	SD_{rel} (%)	% of mean
Ambient temperature			
1 h (n=1)	83 555	–	100
24 h	81 147	2.88	97.41
48 h	76 479	14.86	91.53
72 h	34 098	7.81	40.81
−20°C			
1 h (n=1)	83 555	–	
3 days	85 817	4.80	102.71
13 days	88 153	0.74	105.50

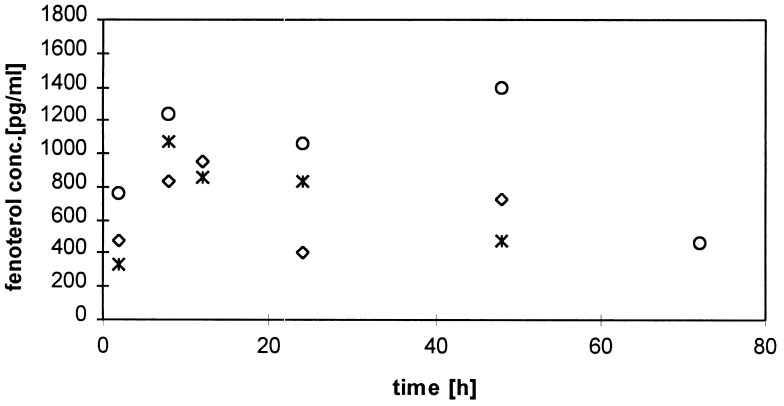

Fig. 6. Fenoterol plasma levels of ∘∘∘ Patient 1, ☆☆☆ Patient 2, ★★★ Patient 3.

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