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A selective HPLC/RIA for the determination of budesonide

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Abstract

A combined HPLC/RIA procedure is described for the selective determination of budesonide (BUD) in plasma. The assay involves the extraction of plasma or serum samples with ethylacetate, consequent HPLC separation of intact budesonide from cross-reacting metabolites on a C_8 reversed phase column, collection of the budesonide containing fraction and determination of budesonide immunoreactivity with the budesonide antiserum. The method was accurate, sensitive (IC_{50} value of 0.9 ng ml^{-1}) and reproducible (intra- and inter-day less than 15%) with a limit of quantification of 0.133 ng ml^{-1} (RSD $< 25\%$). The evaluation of a limited number of clinical samples after oral administration of budesonide by both the HPLC/RIA procedure and a direct RIA using the same antiserum differed in average by a factor of 2, with the ratio of HPLC/RIA-RIA results declining as a function of time. Thus, this ratio might be a suitable indicator for probing for the ratio of budesonide and overall metabolites on a semi-quantitative level.

Keywords: HPLC/RIA; HPLC; Radioimmunoassay; Pharmacokinetics; Budesonide

1. Introduction

Budesonide, (22RS)-16α-17α-butylenedioxy-11β-21-dihydroxypregna-1,4-diene-3,20-dione (BUD), is a potent non-halogenated corticosteroid that contains an asymmetric 16α,17α-acetal group [1,2]. The extensive liver biotransformation [3–5] resulting in three potential metabolites (Fig. 1) is responsible for the low oral bioavailability and efficient systemic removal of budesonide. For this reason, budesonide is currently being evaluated for a number of topical forms of administrations, including the oral treatment of inflammatory bowel disease. The low plasma concentration generally observed for the targeted administration of such a high clearance drugs requires highly sensitive and specific analytical techniques. Currently, direct RIA procedures [6,7] or the use HPLC/MS/MS techniques have been proposed for the determination of glucocorticoids in clinical trials [8]. Over recent years, our group has developed several HPLC/RIA methods [9,10], which overcame the problems of low selectivity of direct RIA assays, thereby being equivalent to the

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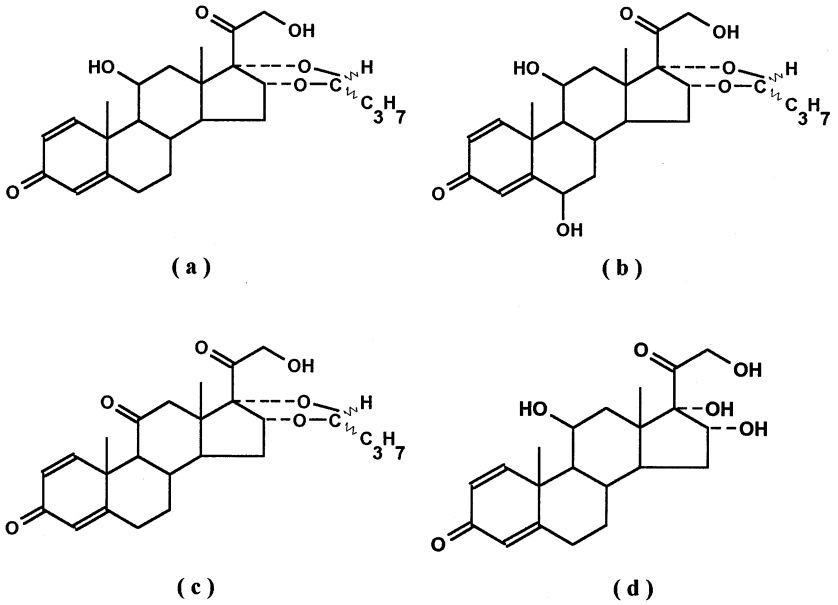

Fig. 1. Chemical structure of budesonide and its major metabolites: (a) budesonide; (b) 6β-hydroxybudesonide; (c) 11-dehydrobudesonide; (d) 16α-hydroxyprednisolone.

rather expensive LC/MS/MS technology with respect to sensitivity and selectivity. Thus, an HPLC/RIA assay for budesonide is reported in this study for the determination of budesonide in the pg ml^{-1} range.

2. Experimental

2.1. Chemicals

BUD and the potential metabolites (11-dihydro-budesonide, 16α-hydroxyprednisolone and 6β-hydroxy-budesonide) were obtained from Sicor (Milano, Italy). 1,2(n) ^{3}H-Budesonide (34 Ci mmol^{-1}) was custom synthesized by New England Nuclear Research (Wilmington, DE). Ethyl acetate and methanol (both HPLC grade) were received from Fisher (Springfield, NJ). Scintillation liquid (CytoScint ES) was purchased from ICN (Costa Mesa, CA), while all other chemicals were of analytical grade and obtained from Sigma (St. Louis, MO). Budesonide antiserum was obtained from Elias USA (Osceola, WI). This budesonide-antigen was synthesized by linking budesonide 3-(O-carboxymethyl)oxime in position 3 to albumin using the mixed anhydride method and immunization was performed in pigs.

2.2. Instrumentation

The HPLC system consisted of a Constametric IIIG pump (LCD/Milton Roy, St. Petersburg, FL), a Perkin-Elmer model ISS-100 auto-injector (Perkin-Elmer, Norwalk, CT), a C_8 reversed phase column (Nucleosil C_8, Keystone, 5 μm, 15 cm × 4.6 mm), a Spectromonitor D variable UV-Detector (LCD/Milton Roy), and a programmable Gilson model 203 fraction collector (Gilson, Middleton, WI). Organic extracts were concentrated on a RC 10-10 concentrator evaporator (Jouan, Saint-Herblain, France), and a Beckman LS 6500 scintillation counter (Beckman Instruments, Fullerton, CA) was used for radioactivity measurements.

2.3. Standards and samples processing

Stock solutions (1 mg ml^{-1}) of BUD were prepared in methanol. For the calibration curve

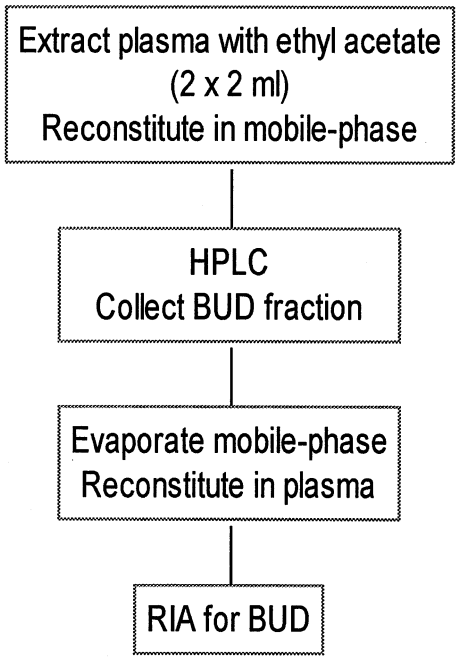

Fig. 2. Scheme of the overall assay procedure: Plasma samples were extracted with ethyl acetate, reconstituted in mobile-phase and injected onto the HPLC system. The budesonide (BUD) fraction was then collected, evaporated and the residue reconstituted in plasma for the RIA.

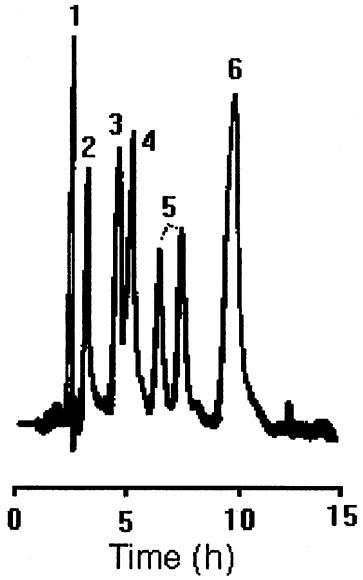

Fig. 3. Chromatogram showing the separation of BUD from its metabolites and hydrocortisone: 1. Solvent front; 2. 16α-hydroxyprednisolone; 3. hydrocortisone; 4. 6β-hydroxybudesonide; 5. 11-dehydrobudesonide; 6. budesonide.

dilutions were prepared in blank plasma to cover the range of 0.066–13.32 ng ml^{-1}. Quality controls of 0.15, 0.60 and 2.0 ng ml^{-1} were prepared from a separate stock solution. Quality control and calibration curve samples were stored in aliquots at −20°C.

KH_2PO_4 (1.7 g) and sodium azide (125 mg) were dissolved in 125 ml of water. Na_2HPO_4 (7.1 g) and sodium azide (500 mg) were dissolved in 500 ml of water. The KH_2PO_4 solution was added to the solution of NaH_2PO_4 until a pH of 7.4 was reached (phosphate buffer). Five-hundred milliliters of this solution and 500 μl of a 1% Triton X solution (1 ml in 100 ml phosphate buffer) were mixed and stored in the refrigerator (assay buffer).

Antiserum (1 ml lyophilized by the manufacturer) was dissolved in 10 ml of water and portions of 500 μl were frozen (antiserum-stock solution). This stock solution (63.4 μl) was diluted with 19 ml of assay buffer and used directly in the RIA. This solution represented consequently a 3000-fold dilution of the original serum.

A dextran coated charcoal suspension (1% Norit A, 0.1% Dextran T70) in phosphate buffer was used to separate bound and unbound steroid. Dextran T-70 (0.05 g) was dissolved in water (500 ml), charcoal (0.5 g) was added and the suspension was mixed for 15 min using a magnetic stirrer. Subsequently the suspension was treated in a sonicator bath for 30 min. The suspension was stored in the refrigerator for up to 1 month.

Radiolabeled 1,2(n)^{3}H-Budesonide (34 Ci $mmol^{-1}$) was stored at −20°C. Ten microliters of this solution (1 Ci ml^{-1}) was added to 500 μl of ethanol and stored at −20°C for up to 3 months. Sixteen microliters of this solution were added to 15 ml of assay buffer and used for the RIA. One-hundred microliters of this dilution contain 3000 cpm (counts per minute).

2.4. Extraction

Aliquots of 500 μl of plasma dilutions (calibration curve and quality controls) were extracted with 2.0 ml of ethyl acetate; the mixture was agitated for 10 min, followed by a 10-min centrifugation at 2000 rpm (Dynac II, Clayton Adams). After removal of the supernatant, the

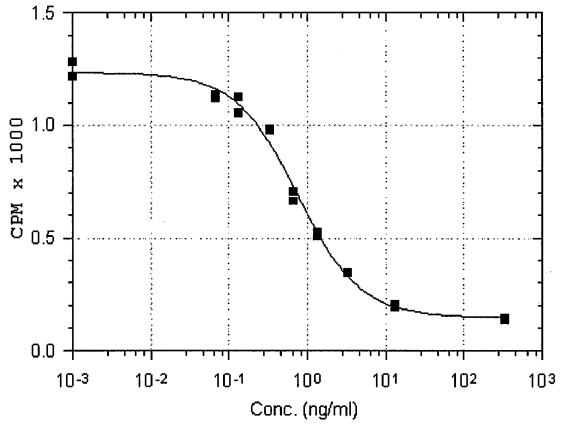

Fig. 4. Calibration curve in human plasma.

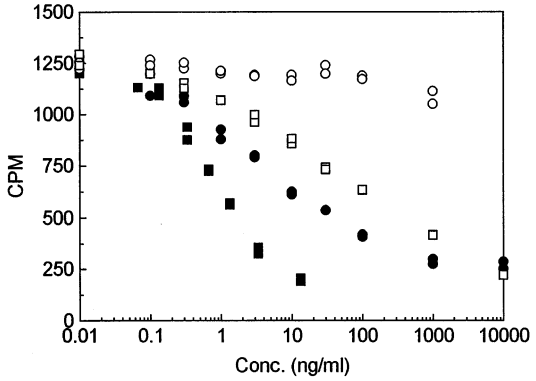

Fig. 5. Cross reactivity of budesonide. (■) Budesonide; (●) 11-dehydrobudesonide; (□) 6β-hydroxybudesonide; (○) 16α-hydroxyprednisolone. Duplicate determinations are shown.

extraction was repeated and the combined organic phases were evaporated. The resulting residues were reconstituted in 500 μl of methanol/water (62.5:37.5, v:v), vortexed for 1 min, followed by a 5-min centrifugation. Aliquots of the supernatant were used for the HPLC separation. To calculate the recovery after extraction, ten blank plasma samples were spiked with ^{3}H-BUD, and extracted with ethyl acetate and 3 consecutive days and reconstituted in blank plasma to the original volume. The extracted radioactivity was then compared with the amounts present in non-extracted plasma.

Table 1
Mean ± standard deviation of the binding parameters of ten calibration curves are listed

	Mean	S.D.	CV (%)
T (cpm)	808	49	6.2
N	1.12	0.09	8.1
IC_{50} (ng ml^{-1})	0.92	0.10	10.7
NS (cpm)	179	23	12.7

cpm: counts per minute; IC_{50}: concentration necessary to reduce specific binding by 50%; *N*: Hill slope factor; *NS*: non-specific binding. T: total specific binding.

2.5. HPLC

The mobile phase was a mixture of methanol/water (62.5:37.5, v:v) with a flow rate of 0.8 ml min^{-1}. UV-Detection was performed at 254 nm. A standard solution of BUD was used to select the time-window to collect the BUD fraction. Two-hundred microliters of the extracted and reconstituted samples were injected onto the column and a 8.7–12.2-min window was used to collect the BUD fraction. These fractions were evaporated, reconstituted in plasma and used in the RIA. Preliminary experiments (see Results) insured that budesonide was sufficiently separated from potential metabolites.

2.6. RIA procedure

Phosphate buffer (250 μl, pH 7.4, 100 mM) was mixed with 100 μl of the anti-serum (diluted 3000 fold), 50 μl of the sample dilution (calibration curve and quality controls) and 100 μl of labeled BUD (^{3}H-BUD 3000 CPM) were mixed and incubated at 4°C for 24 h. Bound and unbound radioactivity were separated by incubation with 100 μl dextran-coated charcoal (0.1% Dextran T-70, 1% charcoal) for 5 min followed by centrifugation at 10000 g (Fisher Microfuge). The radioactivity (CPM) was measured in 500 μl of the supernatant. Data were fitted with the non-linear curve fitting procedure MINSQ (Micro-Math, Salt Lake City, UT) using the logistic function Eq. (1).

$$B = T - \frac{TC^N}{(C^N + IC_{50}^N)} + NS \quad (1)$$

Table 2
Assay range

Nominal conc. (ng ml^{-1})	Mean conc. (ng ml^{-1})	S.D. (ng ml^{-1})	CV (%)	Accuracy (% of nominal)
0.133	0.151	0.03	22.3	112.6
0.333	0.316	0.04	12.9	92.7
0.666	0.636	0.10	16.3	95.9
1.33	1.26	0.13	10.7	94.8
3.33	3.45	0.44	12.8	103.6
13.3	14.7	3.0	20.4	110.6

Two calibration curves were assayed on a given day.
One calibration curve was used for the determination of the binding parameters. The other set was treated as unknown samples. The resulting concentrations are listed and also expressed as percent of the nominal concentrations. The average of the eight independent experiments is given.

Table 3
Intra-day variability of the quality controls

Nominal conc. (ng ml^{-1})	S1	S2	S3	S4	S5	Mean conc. (ng ml^{-1})	S.D. (ng ml^{-1})	CV (%)	Accuracy (%)
0.15	0.14	0.19	0.14	0.12	0.17	0.15	0.03	18.3	101.3
0.60	0.66	0.64	0.64	0.65	0.51	0.62	0.06	10.1	104.2
2.0	1.8	1.8	1.7	2.1	2.0	1.9	0.17	8.9	95.0

Five samples of each concentration were assayed on the same day.

Table 4
Inter-day variability of the quality controls

Nominal conc. (ng ml^{-1})	avg1	avg2	avg3	avg4	avg5	Mean conc. (ng ml^{-1})	S.D. (ng ml^{-1})	CV (%)	Accuracy (%)
0.15	0.14	0.14	0.18	0.13	0.15	0.15	0.02	14.3	98.9
0.60	0.51	0.55	0.58	0.69	0.62	0.59	0.07	11.9	98.3
2.0	2.1	2.1	1.8	2.0	1.9	2.0	0.12	6.1	99.0

Five samples of each concentration were assayed every day on 5 diferent days.

with B, bound tracer in the presence of competitor (CPM); T, total specific binding (CPM in the absence of competitor minus non-specific binding); C, competitor concentration; N, Hill slope factor; NS, non-specific binding (CPM). By knowing the estimates of T, N, NS and IC_{50} (ng ml^{-1} of plasma), the concentrations of the unknown (C_x) was consequently derived from:

$$C_x = IC_{50}^{N} \cdot \left(\frac{100 - \%B}{\%B} \right)^{1/N} \quad (2)$$

with $\%B$, specific binding in the presence of competitor (expressed as percent of the total specific binding T). For the assay characterization, uniform sets of calibration curves were derived at different days. Raw data were used to determine the relevant binding curve parameters (N, T, IC_{50}, NS, Eq. (1)). In order to determine the sensitivity and reproducibility, two calibration curves were analyzed at the same time and the second calibration curve (CPM) was treated as unknown; concentrations were calculated using the first calibration curve and Eq. (2) to obtain the parameters. The limit of quantification was defined as the concentration for which the inter-day variability was less than 25%. Finally, quality controls covering defined concentrations ranges were used to assess intra- and inter-day variabil-

ity. The percent deviation from the mean and its S.D. were calculated for all sets and used as indicator for intra- and inter-day variability.

For the direct radioimmunoassay, RIA procedures were performed as described above, with the exception that 50 μl of plasma or serum were used directly in the RIA incubation.

3. Results and discussion

The developed HPLC/RIA procedure for BUD involved extraction of plasma samples with ethyl acetate (Fig. 2). After extraction, samples were reconstituted in mobile phase. The BUD containing HPLC fraction was collected, evaporated, reconstituted in plasma and assayed by RIA.

3.1. Recovery after extraction

The inter-day results for the recovery of the samples after the extraction with ethyl acetate was $86.0 \pm 2.4\%$ (3 days) and the intra-day variability was $86.5 \pm 3.9\%$ ($n = 10$).

3.2. HPLC separation

BUD was separated from hydrocortisone and its three major metabolites by reversed phase HPLC using methanol/water (62.5:37.5, v:v) as mobile-phase, a C_8 column and a flow rate of 0.8 ml. The system gave well resolved peaks (Fig. 3)

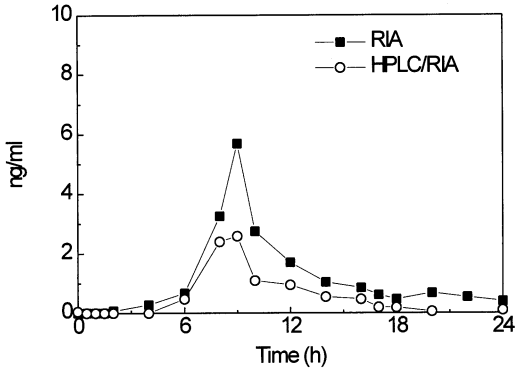

Fig. 6. Serum concentrations after administration of 9 mg of budesonide pH-modified release capsules (Budenofalk) by (○) HPLC/RIA and (■) direct RIA.

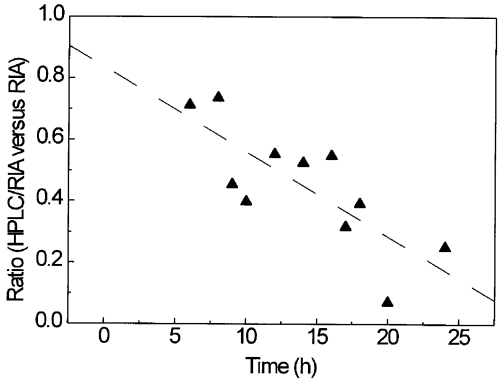

Fig. 7. Ratio of serum levels determined by HPLC/RIA and direct RIA after administration of 9 mg budesonide pH-modified release capsules (Fig. 6).

and eluted BUD after approximately 10 min. The system showed a double peak for 11-dehydrobudesonide probably separating the epimers [3]. A time window of 8.7–12.2 min postinjection was selected for the collection of BUD.

3.3. RIA

A typical calibration curve obtained in the HPLC/RIA is shown in Fig. 4. The assay showed a high affinity of the antiserum to BUD ($IC_{50} = 0.90 \pm 0.13$ ng ml^{-1}) assuring the possibility of monitoring BUD immunoreactivity even at low drug levels. In addition, cross reactivity studies with hydrocortisone were performed. Even at concentrations greater than 10 mg ml^{-1}, hydrocortisone did not modulate tracer binding. The potential metabolite 16α-hydroxyprednisolone also did not bind to the antiserum. The two potential metabolites 6β-hydroxybudesonide ($IC_{50} = 25$ ng ml^{-1}, $N = 0.4$) and 11-dihydrobudesonide ($IC_{50} = 4$ ng ml^{-1}, $N = 0.5$) showed very low affinity to the antiserum. Based on the IC_{50} value of budesonide determined by direct RIA (0.8 ng ml^{-1}), cross reactivities of 16% for 11-dihydrobudesonide and 3.2% for 6β-hydroxybudesonide (Fig. 5) were calculated. These results demonstrated not only a preference of the antiserum to bind to BUD but also the necessity of the HPLC separation due to the high levels of these metabolites in the samples. Binding curve

parameters of ten calibration curves obtained by HPLC/RIA are shown in Table 1. Coefficients of variance (CV) for the binding parameters of total specific binding, N and IC_{50} were smaller than 11%. Non-specific binding showed a CV of 13% and was approximately 20% of the total binding. The Hill slope factor was close to 1, indicating the presence of homogenous binding sites. Table 2 summarizes the accuracy and inter-day variability using calibration curve standards. Accuracy was within 13% and inter-day variability was less than 17% for the concentration range of 0.333–3.33 ng ml^{-1}. The limit of quantification was determined as 0.133 ng ml^{-1} (inter-day variability $< 25\%$). The inter- and intra-day variability of the quality controls (0.15, 0.60 and 2.00 ng ml^{-1}) are shown in Tables 3 and 4. Five samples of each concentration were analyzed on the same day. The experiment was repeated for 5 days. The inter- and intra-day variabilities were lower than 25% for 0.15 ng ml^{-1} and lower than 11% for 0.60 and 2.00 ng ml^{-1}. These results were consistent with the ones shown in the Table 2 for the read-backs of the calibration curves. Thus, this method proved to be specific and sensitive for the measurement of budesonide in plasma in the pg ml^{-1} range. The assay was more sensitive than other HPLC/RIA assays described for dexamethasone [9] and dexamethasone-21-isonicotinate [10], while inter- and intra-day characteristics were comparable. Furthermore, the observed assay characteristics were equivalent to results obtained for a LC/MS method described for budesonide [8].

The assay was consequently applied to serum samples of one volunteer included in a clinical study to evaluate the pharmacokinetics after administration of 9 mg of budesonide in pH modified release capsules. Serum levels were consistent with the delayed release of budesonide from the pH resistant capsules with a time-lag of absorption of about 4 h and a time of maximum concentrations of about 9 h (Fig. 6). Figs. 6 and 7 compare these data obtained by HPLC/RIA with those generated by direct RIA. In average, serum concentrations measured by HPLC/RIA were 50% of those obtained by direct RIA, indicating the higher selectivity of the HPLC/RIA procedure. In addition, there was a significant decrease in the ratio of HPLC/RIA versus RIA results over time after administration of budesonide capsules (Fig. 7). The slope of the resulting correlation was significantly different from zero (test for zero correlation), suggesting that the comparison of HPLC/RIA versus RIA results might be suitable to monitor on a semi-quantitative level a change in the metabolite to drug ratio in pharmacokinetic studies, e.g. for assessing the hepatic metabolic activity in patients with liver disease.

4. Conclusion

In conclusion, the described HPLC/RIA procedure allows the sensitive and specific determination of budesonide in the pg ml^{-1} range. The assay is consequently comparable in sensitivity with the direct radioimmunoassay of budesonide [6], but with the distinct advantage of allowing a selective determination of budesonide (Fig. 6). The assay is reliable and robust and in its present form suitable for processing sample sizes of up to 80 samples per run within a 3-day working unit. The assay might be considered whenever an HPLC/MS/MS methodology is not available.

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