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ANALYSIS OF ALBUTEROL IN HUMAN PLASMA BASED ON IMMUNOAFFINITY CHROMATOGRAPHIC CLEAN-UP COMBINED WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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SUMMARY

A method combining immunoaffinity chromatography with high-performance liquid chromatography was developed for the determination of albuterol in human plasma. The immunoaffinity chromatography, based on the specific interaction of albuterol with the immobilized antibody raised against it, was used as a clean-up step. Albuterol eluted from this immunochemical solid-phase clean-up step was analysed by reversed-phase high-performance liquid chromatography with fluorimetric detection. The performance of the assay was validated on six normal volunteers after a 4-mg oral dose of albuterol, which gave a peak plasma concentration in the range 6.67–15.31 ng/ml at 3–4 h after the dose. Plasma levels (0.79–1.56 ng/ml) of albuterol could be detected up to 24 h after the dose.

INTRODUCTION

Among the selective β_2-adrenergic agonists which are extensively used in the treatment of bronchoconstriction in asthma, albuterol, 2-*tert.*-butylamino-1-(4-hydroxy-3-hydroxymethyl)phenylethanol, is probably the most popular.

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Owing to its hydrophilicity and the low concentrations found in plasma, the extraction of this drug free from interfering compounds is relatively difficult. Numerous assays, including gas chromatography–mass spectrometry [1], high-performance liquid chromatography (HPLC) with fluorimetric and electrochemical detection [2–4], high-performance thin-layer chromatography [5] and radioimmunoassay [6], have been proposed. All these procedures require laborious extraction steps using a solid-phase technique with Sep Pak [7] or XAD2 cartridges [8], repeated liquid–liquid extractions [1], or ion-pair extraction with heptanesulphonate [9] or bis(2-ethylhexyl)-phosphate [3]. Even then, the chromatograms exhibit a narrow zone for the elution of albuterol among other interfering peaks. We propose here an immunoaffinity chromatographic (IAC) approach for the extraction of albuterol, combined with reversed-phase HPLC with fluorimetric detection for the determination of this drug in human plasma samples.

EXPERIMENTAL

Materials

Albuterol hemisulphate was purchased from Sigma (Cat. No. S 5013, St. Louis, MO, U.S.A.). [^{3}H]Albuterol was custom-labelled by Negev Nuclear Research Center (Beer Sheva, Israel) with a specific radioactivity of 17.1 Ci/mmol. The stock solution of albuterol was prepared in distilled water at a concentration of 0.64 mg/ml (as base); it was used to prepare the plasma standards containing 0.8, 1.6, 3.2 and 6.4 ng/ml albuterol. Cyanogen bromide (CNBr)-activated Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). Methanol and acetonitrile (HPLC-grade) were purchased from Burdick & Jackson (Muskegon, MI, U.S.A.), phosphoric acid was obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). All other reagents used were reagent grade unless otherwise specified.

Preparation of the immunoaffinity matrix for albuterol extraction

Preparation of the immunoglobulin G (IgG) anti-albuterol. Antisera against the *o*-(3-carboxypropionyl) derivative of albuterol covalently linked to bovine serum albumin were developed as previously described [10]. Total serum IgG was obtained from serum of immunized rabbits by sodium sulphate precipitation. Briefly, sodium sulphate was added to the serum at a final concentration of 180 mg/ml. After 30 min agitation, the precipitated IgGs were collected by centrifugation (2000 *g*, 10 min). The pellet was washed twice with a solution of sodium sulphate (180 mg/ml) in distilled water. Finally, isolated IgGs corresponding to 10 ml of antisera were resuspended in 10 ml of 0.15 *M* sodium chloride.

Immobilization of IgG to Sepharose 4B. The coupling of IgG with CNBr-activated Sepharose 4B was performed according to the procedure of Gardner

et al. [11], using the above solution in a final ratio of 9.5 mg of polyclonal IgG per g of activated gel. The gel was then washed thoroughly with distilled water followed by alternate washings with 0.1 *M* Tris–HCl buffer (pH 8) to block unreacted isourea groups and 0.1 *M* sodium acetate (pH 4) containing 0.5 *M* sodium chloride. The coupled gel was finally resuspended in 50 m*M* phosphate buffer (pH 7.5) containing 0.02% (w/v) sodium azide and stored at 4°C.

Immunoaffinity chromatography of albuterol. Plasma standards (1 ml) containing 0.8, 1.6, 3.2 or 6.4 ng/ml albuterol and plasma samples (1 ml) from six normal volunteers, collected on 4 m*M* EDTA and withdrawn at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, 16 and 24 h following a 4-mg oral dose of albuterol, were applied on polypropylene Econo columns (Cat. No. 731 1550, Bio-Rad, Richmond, CA, U.S.A.) containing fractions of 0.1 ml of IgG anti-albuterol covalently linked to Sepharose gel. After adsorption, the gel was washed with three 1-ml portions of 50 m*M* phosphate buffer (pH 7.4) followed by two 0.5-ml portions of deionized water. The immunoadsorbed albuterol was then eluted with 2 ml of 0.1 *M* acetic acid containing 20% (v/v) ethanol. The eluate was collected and evaporated to dryness in a Speed Vac concentrator (Savant, Farmingdale, NY, U.S.A.). The residues were reconstituted with 150 μl of the mobile phase, and 100 μl were injected into the HPLC system.

HPLC instrumentation and chromatographic conditions

The high-performance liquid chromatograph consisted of an M 510 pump (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne injector (Model 7125, Rheodyne, Berkeley, CA, U.S.A.), a reversed-phase analytical column (Ultrasphere ODS, 5 μm, 25 cm$\times$4.6 mm I.D., Beckman, Palo Alto, CA, U.S.A., Part No. 235239) and an 2π steradian flow cuvette spectrofluorometer (Model FS 970, Kratos Schoeffels, Westwood, NJ, U.S.A.) set at the following operating conditions: excitation wavelength, 203 nm; no emission filter; attenuation, 0.05 mA; time constant, 6 s. The detector was connected to an electronic integrator (Model C-R3A, Shimadzu, Kyoto, Japan) with the flow chart speed set at 2.5 mm/min. The mobile phase was 0.15% (v/v) phosphoric acid–acetonitrile–methanol (76:14:10, v/v). It was degassed in an ultrasonic bath and purged with helium throughout the chromatographic process. The flow-rate was set at 0.6 ml/min.

HPLC–radioimmunoassay immunograms

In order to assess the specificity of the assay, HPLC–radioimmunoassay immunograms were obtained. Following the HPLC of the serum extracts described above, 0.5-ml fractions of the eluate were collected using a fraction collector (FRAC 100, Pharmacia LKB, Bromma, Sweden). The pH of the eluate was brought to pH 7.4 with 50 μl of 0.5 *M* Tris buffer (pH 8). Aliquots (100 μl) were collected for the radioimmunoassay of albuterol [6].

Calculations

Standard curves were prepared by plotting the peak height (mm) as a function of albuterol concentration (ng/ml) and determining the best-fit line by least-squares linear regression analysis.

Validation of immunoaffinity extraction procedure

Pooled human plasma samples (1 ml) spiked with a mixture of 88 000 cpm [^{3}H]albuterol and 12.8 ng/ml albuterol were applied to 0.1 ml of IgG anti-albuterol covalently linked gel. After the adsorption had been completed, the gel was washed with three 1-ml portions of 50 m*M* phosphate buffer (pH 7.4) and two 0.5-ml portions of deonized water. Various eluting conditions using different organic solvents have been applied. The pass through and the eluates following the washing and elution steps were collected for the radioactivity counting.

Assay validation

The precision of the assay was assessed by testing blank plasma samples spiked with three concentrations of albuterol (0.8, 3.2 and 6.4 ng/ml), analysed in triplicate over five consecutive days. The accuracy of the assay was tested by using 24 blind plasma samples containing 2.13 and 8.53 ng/ml albuterol. The performance of the assay was tested with six normal volunteers following a 4-mg oral dose of albuterol. Plasma samples were collected as described above and frozen at $-20\,^{\circ}$C until analysis.

RESULTS

Evaluation of the immunoaffinity extraction step

The adsorption of albuterol (up to 12.8 ng/ml of plasma) was efficient with the use of 0.1 ml of gel, corresponding to 0.27 mg of immobilized IgG on Sepharose bead, because no leakage occurred during the application of the sample or the washing steps. Following the extensive washing steps to remove endogenous interferences, the desorption of albuterol from the gel was performed by lowering the surface tension of aqueous medium by addition of various amounts of water-miscible organic solvents, such as 2-propanol, ethylene glycol, dimethyl sulphoxide and ethanol. Table I shows the recovery of adsorbed tritiated albuterol on the gel under various elution conditions. The elution medium selected was 0.1 *M* acetic acid containing 20% ethanol. This gave a high and constant recovery of albuterol (mean $\pm$ R.S.D. $= 96.1 \pm 1.11\%, n = 8$) and could be easily evaporated to dryness without any residue for subsequent HPLC analysis. Increasing the ionic strength had only limited influence on the elution of adsorbed tritiated albuterol on the gel, implying that the electrostatic bonds that could mediate the specific interaction between the bound ligand and the immobilized antibody might be minimal. The potential influence of

TABLE I

RECOVERY OF TRITIATED ALBUTEROL IN SPIKED PLASMA SAMPLES ADSORBED ON THE IMMUNOAFFINITY GEL

Elution conditions	Recovery[a] (%)
0.5 *M* Ammonium acetate buffer (pH 4) + 10% 2-propanol	27.8
0.5 *M* Ammonium acetate buffer (pH 4) + 20% ethylene glycol	19.2
0.5 *M* Ammonium acetate buffer (pH 4) + 20% dimethylsulphoxide	63.8
0.5 *M* Ammonium acetate buffer (pH 4) + 10% ethanol	8.1
0.5 *M* Ammonium acetate buffer (pH 4) + 0.5 *M* NaCl	0
0.5 *M* Ammonium acetate buffer (pH 4) + 20% ethanol	85.5
0.5 *M* Ammonium acetate buffer (pH 4) + 20% ethanol	96
0.1 *M* Acetic acid + 20% ethanol (mean ± R.S.D., $n=8$)	96.1 ± 1.11

[a]The determinations were done in duplicate.

metabolites on the immunoaffinity extraction step was investigated by testing the recovery of tritiated albuterol added to blank serum and to serum containing metabolites obtained by mixing aliquots of serum samples from the volunteer after a 4-mg oral dose of albuterol. No difference in the recovery of the radioactivity was found between the two series of samples (mean ± S.E. = 94.5 ± 1.79%).

Chromatography

Fig. 1 depicts typical chromatograms of albuterol in distilled water at a concentration of 6.4 ng per 100 μl (A), a blank plasma extract (B), an plasma extract spiked with albuterol at 6.4 ng/ml (C), a plasma extract from a patient after a 4-mg oral dose of albuterol using IAC (D) and an extract of the same sample using Sep Pak C_{18} cartridge (E). The unique peak from spiked plasma samples extracted by IAC has a retention time of 8.6 min, corresponding with that of albuterol standard. The chromatogram of the plasma extract from the bioavailability study depicted two peaks, eluting at 5.6 and 8.3 min, respectively. The peak at 5.6 min might correspond to the main metabolite of albuterol, which is the 4′-O-sulphate ester derivative [12–14] co-extracted with albuterol in the immunoaffinity step.

The linear regression curve between peak heights and albuterol concentrations over the range 0.8–6.4 ng/ml albuterol is given by the following equation: $y_{\text{estimated}} = 11.7566x + 1.6769$ ($r^2 = 0.992$, $P < 0.01$). The confidence intervals at 99% for the slope factor (β_1) and the intercept (β_0) were $11.240 < \beta_1 < 12.273$ and $-0.228 < \beta_0 < 3.581$. The absolute analytical recovery estimated by comparing the peak height of albuterol extracted from plasma samples with that of a standard sample injected directly into the HPLC system is 80.14 ± 5.33% (mean ± S.D.) over the concentration ranges studied. Because of good recov-

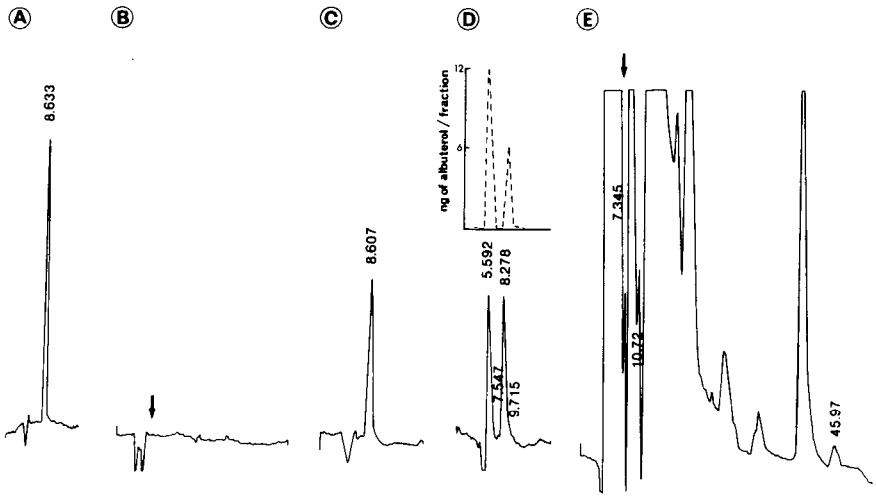

Fig. 1. Typical chromatograms of (A) albuterol aqueous standard (9.6 ng albuterol), (B) blank human plasma extract, (C) human plasma extract spiked with albuterol at 6.4 ng/ml, (D) plasma extract from patient sample after a 4-mg oral dose of albuterol using IAC (concentration detected at 6.4 ng/ml), and (E) plasma extract using Sep Pak cartridge. The inset in (D) shows the immunogram obtained with fractions collected after HPLC separation, expressed as nanograms of immunoreactive albuterol detected per fraction.

TABLE II

EVALUATION OF THE ACCURACY OF THE ASSAY

Theoretical spiked albuterol concentration (ng/ml)	*n*	Mean absolute error	Mean relative error (%)	Standard error	Relative standard deviation (%)
2.13	12	0.2	9.39	0.03	4.81
8.53	12	0.04	0.98	0.05	2.20

ery and the use of a constant-volume injection loop, the use of an internal standard was found unnecessary.

Validation of the assay

The accuracy of the assay was tested by using blank plasma to which albuterol was added to give concentrations of 2.13 and 8.53 ng/ml. The results are shown in Table II. The precision of the assay was assessed by analysing in triplicate blank plasma spiked with 0.8, 3.2 and 6.4 ng/ml albuterol over five consecutive days. The intra-day assay coefficients of variation (C.V.) were 9.5, 4.7 and 5.0%, respectively. The between-day assay C.V. were 8.5, 9.7 and 6.1%, respectively. The detection limit of the assay was 0.5 ng/ml, which was the

smallest concentration of albuterol spiked to plasma that gives a signal-to-noise ratio of 3:1.

Fig. 2 shows the albuterol plasma concentrations observed in six normal volunteers after a single 4-mg oral dose of albuterol. The time of maximum plasma albuterol concentration (I_{max}) was 3–4 h, and the peak plasma concentration (C_{max}) ranged from 6.67 to 15.31 ng/ml. The comparison of radioim-

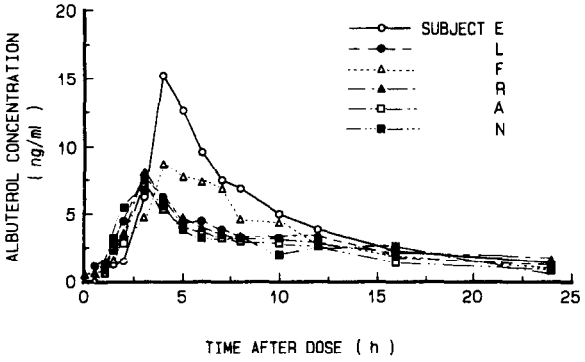

Fig. 2. Plasma albuterol concentrations in six subjects following the administration of a 4-mg oral dose of albuterol.

TABLE III

COMPARISON OF RADIOIMMUNOASSAY AND IAC-HPLC FOR ALBUTEROL IN PLASMA

Data obtained from samples from a volunteer who took a 4-mg oral dose of albuterol.

Sampling time (h)	Concentration (ng/ml)	
	Radioimmunoassay	IAC-HPLC
0	–	–
0.5	2.09	1.15
2	1.69	2.58
1.5	2.57	1.52
2	3.51	3.29
3	11.21	8.19
4	8.82	5.46
5	8.41	5.03
6	7.24	4.84
7	6.13	3.50
8	5.50	2.90
10	4.83	3.04
12	4.90	3.04
16	4.18	2.33
24	2.85	1.23

munoassay [6] and IAC–HPLC assay for albuterol in plasma from a volunteer after a 4-mg oral dose of this drug is shown in Table III.

DISCUSSION

One of the important parts of the development of a new method is the sample clean-up, particularly for hydrophilic residues at low nanogram levels in biological matrices. The strategy of exploiting the characteristic properties of biological molecules, such as IgGs, to bind to specific ligands in a reversible and highly specific manner has been used successfully for the extraction of albuterol free from endogenous interferences.

This very effective sample clean-up is evidenced by comparing the chromatogram obtained following IAC of the plasma sample with that obtained after extraction using by Sep Pak cartridges. With regard to the specificity of the IAC, the immobilized IgGs do not cross-react with ephedrine, isoproterenol, phenylephrine, amphetamine and terbutaline (data not shown), but they do cross-react with the metabolites of albuterol. The co-extraction of albuterol and its metabolite in the IAC step is evidenced by the presence of two peaks, eluted at 5.6 and 8.3 min, respectively, in the chromatogram of the plasma extract. The immunogram obtained with the eluted fractions from HPLC depicts two immunoreactive peaks, corresponding to those detected by fluorimetry. This confirms the identity of the peak eluted at 5.6 min as the albuterol metabolite that cross-reacts in the radioimmunoassay for albuterol. The characterization of this metabolite by acidic or enzymic hydrolysis has not been carried out. It might be the 4′-O-sulphate ester derivative, which is thought to be the main metabolite of this drug in humans, resulting from the first-pass sulphation [12–14]. However, this metabolite is well separated from the parent compound by HPLC.

The comparative data in Table III, show agreement between the values obtained by the two methods ($r=0.94$). However, the values obtained by IAC–HPLC are lower, probably owing to the higher specificity of the present procedure. The IAC–HPLC combination appears to be a powerful tool for ensuring the specificity of the assay for albuterol. The efficiency of the IAC step would allow the handling of a large number of clinical samples without extensive maintenance of the HPLC column and the detection system. Furthermore, the trace enrichment of albuterol from a larger volume of plasma by immunoaffinity extraction might be useful for the pharmacokinetic investigation of albuterol given by inhalation.

In conclusion, the present method of combining specific IAC with the separation and detection capability of HPLC gives adequate reliability and sensitivity for the determination of albuterol in plasma at subnanogram levels. It would be particularly useful in pharmacokinetic studies or clinical monitoring of this drug.

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