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Solid phase extraction of clenbuterol from plasma using immunoaffinity followed by HPLC ☆

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Abstract

An immuno-extraction column for clenbuterol has been prepared. Optimum conditions for the selective retention and elution of clenbuterol have been developed, based on a modification of our earlier work on morphine, chlortoluron and isoproturon. Clenbuterol could be retained on the immuno-column then eluted in one x one ml fraction using 50% methanol in phosphate buffered saline pH 2. On columns containing antisera (but not to clenbuterol) the clenbuterol was removed in the washing step. HPLC–UV determination gave clean traces. Day-to-day reproducibility was improved by precipitating the plasma proteins with acetonitrile. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The determination of drugs at low concentrations in biological samples is a very important and exacting task. These assays are carried out for a number of reasons including pharmacokinetic and metabolism studies, therapeutic drug monitoring, compliance testing, forensic analysis, toxicology, residue analysis and doping control in sport. Many developments in analytical instrumentation are geared to fulfilling the requirement for specific, sensitive, reliable, cost-effective analysis. Much effort is devoted to increasing the efficiency of separations (theoretical plates) or the development of universal or specific sensitive detectors.

It is generally recognised however that when dealing with biological samples direct introduction into chromatographic instruments is undesirable and that some form of sample preparation is necessary. Furthermore it is also recognised that sample preparation is the part of the analytical procedure most likely to contribute to analytical uncertainty. The advent of automated injection and data handling as routine on modern chromatographic instruments means that sample preparation is the most time consuming, labour intensive and therefore costly part of the procedure when dealing with complex matrices.

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Despite its importance relatively little research effort is devoted to sample preparation. We have been investigating the possibility of using antibodies immobilised onto silica or controlled pore glass as highly selective solid phase extraction columns. This we have successfully carried out for the herbicides chlortoluron [1] and isoproturon [2] separately and for a range of phenylureas on a mixed bed of the two immuno-sorbents [3,4]. The procedures developed demonstrated the feasibility of using this approach for environmental samples such as waters. We have recently extended the approach to show the possibility of selectively extracting the drug morphine from urine [5]. Our work has been aimed at demonstrating that retention was based almost entirely on antibody–antigen interaction thus enabling elution from the immuno-sorbent in a low (usually 1 ml) volume. Other examples of immuno-extraction followed by chromatography include steroids [6,7], chloramphenicol, [8] cannabinoids [9] thromboxane metabolites [10], propranolol [11], clenbuterol [12], phenylureas and atrazine [13].

The work described below shows the optimisation of an immuno-extraction procedure for the drug clenbuterol and the feasibility of using such a procedure for the determination of clenbuterol in plasma. The aim was to apply our generic protocol for immuno-extraction with minimal modification to assess the feasibility of using it for extraction and clean-up of a drug in plasma. Clenbuterol is a β-sympatomimetic or β-agonist drug frequently used for the treatment of obstruction in the bronchial tubes of animals. It is also used as a growth promoter in animals and as a performance enhancing (illegal) drug in sport. Methods to assay clenbuterol include GC–MS [14,15], and HPLC [16]. It was chosen as a model compound as most procedures require extensive sample preparation and as antisera was available.

2. Experimental

2.1. Chemicals

Clenbuterol was obtained from Sigma Chemicals, Poole, UK. Ethanol, methanol, acetonitrile, glacial acetic acid, hydrochloric acid, triethylamine, disodium hydrogen phosphate, sodium chloride, sodium dihydrogen phosphate, potassium dihydrogen phosphate and potassium chloride were from BDH-Merck, Poole, UK. All reagents were AR grade or equivalent. Phosphate buffered saline (PBS) was prepared by dissolving sodium chloride (8 g), potassium dihydrogen phosphate (0.2 g) and disodium hydrogen phosphate (0.2) in water (1 l). The pH of PBS was adjusted to pH 7 using ortho phosphoric acid.

2.2. Chromatographic conditions

The HPLC pump was a Beckman 110B (Beckman Instruments, High Wycombe, UK) operated at 1 ml min^{-1}. UV detection was performed with a Pye Unicam (Cambridge, UK) detector set at 230 nm. The HPLC mobile phase was 25% acetonitrile, 74% 0.05 sodium dihydrogen orthophosphate and 1% triethylamine adjusted to pH 2.5 with orthophosphoric acid. The column was Spherisorb ODS-2 (Jones Chromatography, Hengoed, UK) 5 μm, 150 × 5 mm I.D. Injection was 100 μl using a WISP (Waters Associates, Northwich, UK).

2.3. Immuno-columns

The antisera was obtained from the School of Biological Sciences, University of Surrey. Polyclonal antibodies were raised in rabbit using clenbuterol–BSA. Further details are available [17]. Clenbuterol immuno-columns were prepared by Clifmar Associates, Guildford, UK. A polypropylene disposable separation column was packed with aldehyde activated silica (1.2 g wet weight). The column was washed with 50 ml of PBS to remove the remaining traces of gluteraldehyde in the solid phase. Next 5 ml of PBS buffer was dispensed into the column followed by 500 μl clenbuterol antisera. The column was closed at both ends and rolled on a rotamixer for 2 h. The column was then washed with 10 ml PBS. A 5-ml aliquot of 1 M glycine buffer was added and the column left overnight. The next day the column was washed with 10 ml of 0 036 M hydrochloric acid followed by 20 ml of PBS.

2.4. Extraction protocol

Clenbuterol stock solution was 1 mg ml^{-1} in methanol. A working standard of 1 μg ml^{-1} in methanol was used and diluted further in the appropriate solvent. For plasma standards measured quantities were evaporated to dryness and re-dissolved in the appropriate volume of control human plasma. Typical experiments consisted of loading clenbuterol (1 ml of 200 ng ml^{-1}) onto the immuno-columns in PBS (pH 7), washing the column with 5–10 ml of PBS (pH 7) and then eluting in PBS at low pH containing organic modifier. Fractions (1 ml) were collected during loading (known as breakthrough), washing and elution. These fractions were then analysed by HPLC.

Once the optimised washing and elution protocol had been obtained using clenbuterol solutions in buffer, plasma samples were investigated. The optimised protocol for clenbuterol was:

1. Load the column with 0.1 ml plasma diluted with 0.9 ml of PBS
2. Wash with 20 ml of PBS
3. Elute with 2 × 1 ml of 50% methanol in PBS, pH 2, collect the second fraction
4. Inject 100 μl onto HPLC.

For protein precipitation 1 ml of acetonitrile was added to 0.1 ml plasma. The sample was vortex mixed, centrifuged and the supernatant collected. This was evaporated to dryness under nitrogen and re-dissolved in 1 ml PBS before loading on the immuno-column.

3. Results and discussion

HPLC–UV was based on a published procedure [18]. The main objective of the work was to develop and optimise a procedure to retain clenbuterol on the immuno-column and then to elute in a small fraction, preferably 1 ml. Loading the column with 200 ng of clenbuterol showed no drug in the breakthrough or 10 × 1 ml washing fractions (PBS pH 7 or distilled water). This indicated that clenbuterol was retained on the column under these conditions. Our previous work [1,2,5] had shown that lowering the pH of the PBS and adding organic modifier allowed elution of other analytes. Table 1 (top third) shows the recovery of clenbuterol in PBS at different pH. The results show that the best recovery of clenbuterol occurs at low pH. Even at pH 2 it was not possible to obtain quantitative recovery of

Table 1
The recovery of clenbuterol (200 ng) at different elution buffer composition

Elution buffer (PBS)	Elution fraction 1	Elution fraction 2	Elution fraction 3	Elution fraction 4	Total recovery
pH 7	0	0	0	0	0
pH 6	0	0	0	0	0
pH 5	0	0	11	10	21
pH 4	0	3	18	21	42
pH 3	0	0	16	26	42
pH 2	0	13	68	11	92
10% MeOH pH 7	7	7	10	0	24
20% MeOH pH 7	13	0	0	17	30
30% MeOH pH 7	7	7	17	13	44
40% MeOH pH 7	0	7	27	10	44
50% MeOH pH 7	0	10	50	23	83
50% MeOH pH 7	0	43	32	10	85
50% MeOH pH 6	2	50	35	13	100
50% MeOH pH 5	5	35	38	10	88
50% MeOH pH 4	0	13	50	8	71
50% MeOH pH 3	0	50	15	3	68
50% MeOH pH 2	0	108	0	0	108

Table 2
The recovery of clenbuterol (200 ng) from different sorbents[a]

Sorbent	Break through	W1	W2	W3	W4	W5	E1	E2	E3	E4
Anti-clenbuterol	0	0	0	0	0	0	0	108	0	0
Normal sheep serum	0	0	21	41	24	14	0	0	0	0
Anti-morphine	0	0	35	46	19	0	0	0	0	0
Activated silica	0	48	36	29	0	0	0	0	0	0

[a] W are wash fractions, E are elution fractions.

clenbuterol in 1 × 1 ml fraction. Table 1 (middle third) shows the effect of different concentrations of methanol in the elution buffer at pH 7, indicating that quantitative recovery was not possible under any of the conditions tried. Table 1 (bottom third) shows the recovery of clenbuterol at different pH of PBS when using 50% methanol in the elution buffer. Here it can be seen that the desired result was obtained. Quantitative recovery of clenbuterol was obtained in 1 × 1 ml fraction using an elution buffer of 50/50 methanol/PBS at pH 2. This protocol is very similar to those we have developed for morphine [5] which used 40% ethanol in PBS pH 2, and for chlortoluron [1] and isoproturon [2] which both used 50% ethanol in PBS pH 2.

Table 2 shows the recovery of clenbuterol from the column containing clenbuterol antisera, a column containing morphine antisera one containing normal sheep serum and activated silica. As can be seen only the anti-clenbuterol column gives retains the drug during the washing stage, followed by elution in the single 1-ml fraction.

Using the optimised protocol (elution with 50% methanol in pH 2 PBS) the immuno-columns were tested for their ability to clean up clenbuterol spiked into plasma. Now only the second elution fraction (E2 in the tables) was analysed. Plasma samples (0.1 ml) were diluted tenfold with pH 7 PBS before being introduced onto the immuno-column. Experiments were carried out as detailed in the experimental section earlier. Clean HPLC traces were obtained in the clenbuterol region of the chromatogram. Plasma pools 50 and 100 ng/ 0.1 ml plasma were spiked with clenbuterol at three different concentrations. These were analysed over six different days to give an indication of the day-to-day reproducibility of the method. The results showed unacceptably high RSD, up to ±44%. A further experiment to investigate the effect of plasma proteins was carried out. The spiked plasma (0.1 ml) was added to 1 ml of acetonitrile, proteins precipitated and the supernatant evaporated to dryness before re-dissolving in PBS pH 7 and then carrying on with the immuno-extraction protocol. The results were improved. The RSD within-day was ±2.4%, ±4.0%, and ±0.8% at the 50 ng, 100 ng and 200 ng /0.1ml plasma and day-to-day it was ±4.2%, ±2.4%, and ±2.3% respectively at the same concentra-

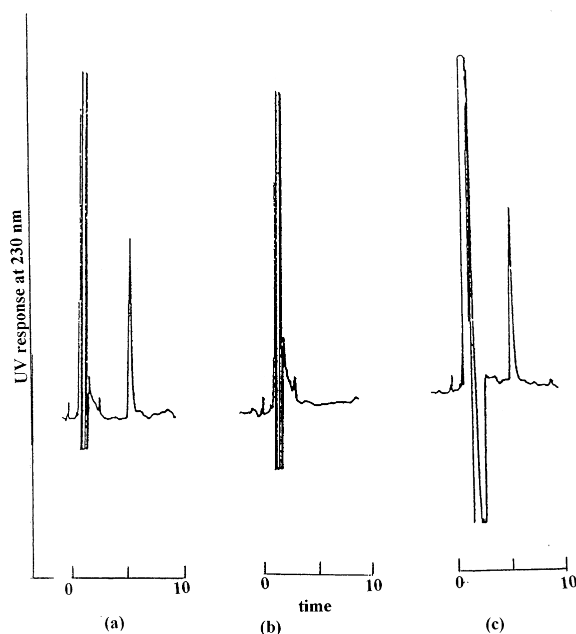

Fig. 1. HPLC of clenbuterol. (a) Unextracted standard, (b) plasma blank, (c) standard after immuno-extraction. HPLC conditions: Column, Spherisorb ODS-2, 5 μm, 150 × 5 mm I.D. Mobile phase 25% acetonitrile, 74% 0.05 sodium dihydrogen orthophosphate and 1% triethylamine adjusted to pH 2.5 with orthophosphoric acid, at 1 ml min^{-1}. Detector UV at 230 nm. Injection 100 μl.

tions. Specimen chromatograms for unextracted standard, plasma blank and clenbuterol immuno-extraction are shown in Fig. 1. The peaks in the chromatogram are almost entirely due to the elution buffer in which the clenbuterol is dissolved.

The capacity of the immuno-columns was monitored throughout this programme. Before use the immuno-columns had a capacity of approximately 300 ng. This had dropped by only 30% after 14 days use and only by half after 50 days use, demonstrating that if necessary columns could be re-used many times. The method was capable of detecting clenbuterol down to at least 100 ng ml^{-1}. Even lower limits could be achieved by pre-concentrating the elution buffer and re-dissolving in mobile phase, or by using a more sensitive detection system such as HPLC–MS. The standard curve was linear up to 250 ng clenbuterol/0.1 ml plasma.

Although the aim of the present work was to investigate the feasibility of immuno-extraction toclean-up biological samples, rather than to develop a new method for clenbuterol, the data produced does suggest that immuno-extraction shows great possibilities for this type of analysis, confirming our earlier work on morphine, isoproturon and chlortoluron.

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