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A radioimmunoassay method for the determination of nedocromil sodium in plasma and urine

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Abstract: A radioimmunoassay method for the determination of nedocromil sodium (FPL 59002 disodium salt) in human plasma and urine is described. The method employs a primary antiserum raised in a sheep, and a mono-tyramide derivative of nedocromil sodium labelled with iodine-125 as a heterogeneous radioligand. Free and bound radioligand are separated using a secondary anti-sheep IgG antiserum. All three reagents are added simultaneously to samples containing nedocromil sodium prior to an overnight incubation. The method has a limit of detection of 0.25 ng ml^{-1}, when plasma sample volumes of 100 µl are analysed, and is accurate and precise. Inter-assay relative standard deviations (N = 18) of 15.1, 5.0 and 5.6% were found at concentrations in plasma of 0.5, 2.0 and 6.0 ng ml^{-1} respectively. The method is specific as indicated by negligible cross-reaction of the anti-nedocromil sodium antiserum with a range of drugs. The method is applicable to the analysis of samples from subjects who have inhaled nedocromil sodium from a pressurised aerosol.

Keywords: *Radioimmunoassay; nedocromil sodium; FPL 59002 disodium salt; ^{125}I-labelled radioligand.*

Introduction

Nedocromil sodium (FPL 59002 disodium salt, disodium 9-ethyl-6,9-dihydro-4,6-dioxo-10-propyl-4H-pyrano[3,2-g]quinoline-2,8-dicarboxylate, I, Fig. 1) is a novel compound synthesised by Fisons which displays selective bronchial anti-inflammatory properties. The compound is undergoing clinical trials in conditions such as reversible obstructive airways disease [1, 2]. In the treatment of these conditions nedocromil sodium is inhaled from a pressurised aerosol (Tilade®) into the lungs.

As with other drugs delivered by inhalation [3] only a small proportion of the administered dose reaches the lungs: the majority of the dose is deposited in the upper respiratory tract and is subsequently swallowed. Although the compound is well absorbed from the lung, it is poorly absorbed from the gastrointestinal tract. Plasma

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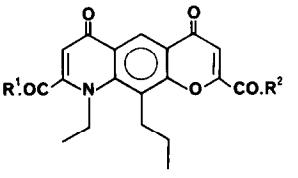
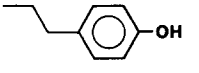


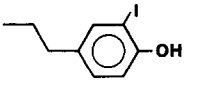
I $R^1 = R^2 = ONa$

II $R^1 = R^2 = Cl$

III $R^1 = ONa$, R^2 = bovine serum albumin

IV R^1 = bovine serum albumin, $R^2 = ONa$

V $R^1 = OH$, $R^2 = NH$— 

VI $R^1 = OH$, $R^2 = NH$— 

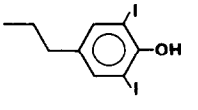
VII $R^1 = OH$, $R^2 = NH$— 

Figure 1
Structure of nedocromil sodium (I) and derivatives.

concentrations of nedocromil sodium in man were therefore expected to be low. In order to support pharmacokinetic and clinical evaluation of the drug, a sensitive analytical method for the determination of the compound in plasma was required, ideally with a limit of detection of 1 ng ml^{-1} or lower. Our previous studies [4] with the anti-allergy agent sodium cromoglycate suggested that radioimmunoassay might prove a suitable technique for the determination of nedocromil sodium. Antisera were therefore raised against a synthetic nedocromil sodium immunogen in sheep.

Initially a radioimmunoassay using [^{3}H]nedocromil sodium as radioligand was developed since high specific activity tritiated drug was easily prepared via a rhodium(III) chloride catalysed exchange procedure [5]. This method was found to possess the required sensitivity with a limit of detection of 0.5 ng ml^{-1}. However, large plasma sample volumes of 300 µl per determination were required. Moreover, the method was found to be somewhat impractical for the analysis of large numbers of samples because of the tedious sample preparation protocol required for counting the ^{3}H-isotope. In addition, complete utilisation of one or more β-scintillation counters was required. To avoid these limitations an alternative radioligand was synthesised. Since nedocromil sodium is not amenable to direct radiolabelling with a gamma-emitting nuclide, a mono-tyramide derivative was synthesised to provide a suitable substrate for radioiodination with iodine-125. The radioimmunoassay developed using the resulting heterogeneous ^{125}I-radioligand is described here. It is more sensitive, more precise, uses less sample and is more convenient than the ^{3}H-radioligand method.

Experimental

Materials

Nedocromil sodium, ambicromil sodium [6], minocromil [7] and sodium cromoglycate are products of Fisons plc, Pharmaceutical Division, Loughborough, UK. Bovine serum albumin (fraction V), tyramine, chloramine-T, phenylbutazone, theophylline, warfarin, chlorpromazine hydrochloride, sodium salicylate, sodium acetylsalicylate, imipramine hydrochloride and sodium prednisolone-21-succinate were purchased from the Sigma Chemical Company, Poole, UK. Other drugs were purchased from the manufacturers. Dibenzo-18-crown-6 was purchased from the Aldrich Chemical Company, Gillingham, UK. Carrier-free sodium [^{125}I]iodide was purchased from Amersham International plc, Amersham, UK. All other chemicals and solvents were purchased from Fisons Scientific Equipment, Loughborough, UK, and were analytical grade quality, if available.

Donkey anti-sheep IgG antiserum was purchased as lyophilised material from the RAST Allergy Unit, Benenden Chest Hospital, Cranbrook, UK. Non-ulcerative Freund's incomplete adjuvant (Morris) was purchased from Guildhay Antisera, Guildford, UK, and BCG vaccine was purchased from Glaxo, Greenford, UK.

Blank human plasma was obtained by the centrifugation of lithium heparin–anti-coagulated blood (100 ml) collected, using microflex infusion sets and disposable syringes (50 ml), from a panel of healthy donor subjects.

Pooled blank human plasma was prepared by mixing equal volumes of plasma from ten or more donors. Blank human urine was collected, without preservative, during a 24 h collection period. All samples were stored at −20°C.

Equipment

Immersible CX-10 ultrafilters were purchased from Millipore (UK) Ltd., Harrow, UK. Disposable pyrex tubes (75 × 12 mm) were purchased from Fisons Scientific Equipment, Loughborough, UK. The Micromedic automated pipetting station (model 24006) with auxiliary pipette (model 25006) were obtained from Chemlab Instruments, Hornchurch, UK. The NE 1600 multiwell gamma counter, which was interfaced to a Commodore 4032 professional computer was obtained from Nuclear Enterprises Ltd., Edinburgh, Scotland, UK.

Methods

Preparation of the nedocromil sodium–bovine serum albumin immunogen. Nedocromil sodium (200 mg) in admixture with a tracer amount of [^{14}C]nedocromil sodium and dibenzo-18-crown-6 (133 mg) were dissolved, by sonication, in *N,N*-dimethylacetamide (7 ml). The solution was cooled on ice and thionyl chloride (666 μl) added in three equal portions over 1 min. The resulting yellow solution of the bis-acid chloride of nedocromil sodium (II, Fig. 1) was maintained at 0°C for a further 12 min and then rapidly mixed with a solution of bovine serum albumin (200 mg) in water (25 ml) containing sodium hydrogen carbonate (4 g). The solution was adjusted to pH 6.5 with aqueous ethanoic acid solution (1:1, v/v). Repeated dialysis of the solution, using an Immersible CX-10 ultrafilter, and lyophilisation yielded the bovine serum albumin conjugates of nedocromil sodium (III and IV, Fig. 1). Estimates from both the ultraviolet absorbance of the conjugate at 390 nm and the specific radioactivity of the immunogen indicated that approximately 5 moles of nedocromil sodium were incorporated per mole of bovine serum albumin.

Production of the anti-nedocromil sodium antiserum. The nedocromil sodium–bovine serum albumin immunogen (1.2 mg) in sterile isotonic saline (4 ml) was homogenised with non-ulcerative Freund's incomplete adjuvant (8 ml) and aqueous BCG solution (0.2 ml), prepared as for intradermal injection, to obtain an emulsion. The mixture was cooled on ice whilst preparing this emulsion. The emulsion (0.5 ml) was injected *intramuscularly* into each of the limbs of three Suffolk-cross sheep. Each animal received 200 μg of the immunogen at each immunisation. Subsequent boosting immunisations, from which the BCG solution was omitted, were performed 31, 59, 107 and 192 days after the priming immunisation. The sheep were bled, generally 7, 10 and 13 days after the boosting immunisation, and the serum was isolated for determination of the anti-nedocromil sodium titre. Large batches of the antisera were collected on days 40, 69, 118 and 207.

Preparation of the mono-tyramide derivative of nedocromil sodium. Nedocromil sodium (500 mg) was converted to the bis-acid chloride derivative (II, Fig. 1) using the procedure detailed above, the amounts of the other reagents used being increased *pro rata.* To the resulting solution, a solution of tyramine (340 mg) in *N,N*-dimethyl-acetamide (4.0 ml) was added over a 5 min period. After stirring for a further 2 h at ambient temperature, the mixture was poured into a solution of sodium hydrogen carbonate (50 g l^{-1}, 150 ml) and extracted with trichloromethane/propan-2-ol (1:1, v/v, 100 ml × 2) to remove the bis-tyramide derivative of nedocromil sodium. The aqueous layer remaining was acidified to pH < 1.0 with concentrated hydrochloric acid and extracted with further portions of trichloromethane/propan-2-ol (1:1, v/v, 100 ml × 2). This organic extract was washed with water (100 ml × 2) and the solvents were removed under reduced pressure to obtain the crude mono-tyramide derivative of nedocromil sodium (V) as a dark brown oil. Thin layer chromatographic analysis (Merck pre-coated silica gel F_{254} TLC plates, ethyl ethanoate/propan-2-ol/water 10:7:6, v/v) of this crude material revealed two main components, the more polar corresponding to nedocromil sodium (R_f 0.60) and the other to the required mono-tyramide derivative (R_f 0.78).

The crude material was purified by reversed-phase HPLC (Spherisorb 5 ODS 250 × 22.5 mm column; methanol/aqueous ammonium ethanoate, 65 mM, 11:9, v/v; 16 ml min^{-1}; 280 nm). Those chromatographic fractions containing the mono-tyramide derivative (retention time ≈12 min, were combined, acidified with hydrochloric acid (2 M) to pH < 2.0, and extracted with trichloromethane/propan-2-ol (1:1, v/v). The organic solvents were removed under reduced pressure and the resulting yellow solid was dried under vacuum to yield the purified mono-tyramide derivative [V, 121 mg, mp 300°C (decomp.), δ (360 MHz), $(C^2H_3)_2SO$) 9.20 (1H, s), 8.83 (1H, t), 8.60 (1H, s), 7.05 and 6.70 (4H, q), 6.86 (1H, s), 6.67 (1H, s), 4.30 (2H, q), 3.50 (2H, m), 3.20 (2H, t), 2.80 (2H, t), 1.75 (2H, m), 0.9–1.0 (6H, 2t); ms (fast atom bombardment, m/z) 491 (M + 1); ms (electron impact, m/z) no M^+, 446, 327, 312, 297, 283]. The structure of the mono-tyramide derivative was assigned as compound V (Fig. 1) rather than the alternative isomer (where R^1 and R^2 of V are reversed) by ^{1}H-NMR studies of the corresponding derivative methyl esters.

Preparation of the radioligand. The radioiodination of the mono-tyramide (V) was performed in the microvial (P15) in which the carrier-free sodium [^{125}I] iodide (37 MBq, approximately 600 MBq μg^{-1}, 3.7 GBq ml^{-1}) was supplied. Aqueous potassium iodide solution (10 μg ml^{-1}, 40 μl) and a solution of V (100 μg ml^{-1}, 50 μl) in sodium

phosphate buffer (0.1 M, pH 7.5) were added to the microvial using microsyringes (100 μl). The radioiodination was initiated by the addition of an aqueous solution of chloramine-T (50 μg ml^{-1}, 40 μl) and the microvial was vigorously shaken. After a reaction period of 1 h, during which time the microvial was shaken several times, aqueous sodium metabisulphite solution (150 μg ml^{-1}, 40 μl) was added. The contents of the microvial were transferred to a glass storage vial using a disposable syringe (1 ml). The microvial and syringe were repeatedly washed with a mixture (7 ml) of sodium phosphate buffer (0.1 M, pH 7.2) and methanol (1:2, v/v). These washings were added to the storage vial with an additional volume (3 ml) of the same mixture of buffer and methanol to prepare the stock solution of the radioligand. Typically 90–95% of the supplied radioactivity was recovered in the stock solution. Analysis of the stock solution by reversed-phase thin layer chromatography (Whatman KC_{18} TLC plates; methanol/ammonium ethanoate, 175 mM, 7:3, v/v) indicated that 88–93% of the radioactivity present in the stock solution of radioligand co-chromatographed with the monoiodo- and diiodo-derivatives (VI and VII, Fig. 1) of V. The stock solution of the radioligand was stored at 4°C and was used in the radioimmunoassay for periods of up to two calendar months.

Preparation of the buffer used in the radioimmunoassay. The sodium phosphate buffer (0.1 M) used in the radioimmunoassay was prepared by adjustment to pH 6.0 of a solution of sodium dihydrogen orthophosphate dihydrate.

Preparation of standard solutions of nedocromil sodium and standard and quality control plasma samples. Hydrated nedocromil sodium (13% water, w/w) was dissolved in the radioimmunoassay buffer to obtain a concentration of the hydrated compound of 100 μg ml^{-1}. The absorbance at 253 nm of a ten-fold dilution, in the buffer, of this solution was determined and compared with the absorptivity (1% w/v, 1.0 cm) of anhydrous nedocromil sodium (E_1^1 = 878) to allow calculation of the concentration of anhydrous nedocromil sodium present in the solution of the hydrated compound. The solution of hydrated compound (100 μg ml^{-1}) was appropriately diluted in the buffer to obtain a stock standard solution of anhydrous nedocromil sodium (10 μg ml^{-1}). Working standard solutions at concentrations of 25, 50, 100, 200, 400, 600 and 800 ng ml^{-1} were prepared from the stock standard solution by dilution in buffer. Standard plasma samples at concentrations of 0, 0.25, 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 ng ml^{-1} were prepared by the addition, to pooled blank human plasma (9.9 ml), of buffer (0.1 ml) or of the working standard solutions (0.1 ml). Quality control plasma samples at concentrations of 0.5, 2.0 and 6.0 ng ml^{-1} were similarly prepared using larger appropriate volumes of pooled blank human plasma. All samples were stored at −20°C.

Preparation of the solutions used in the radioimmunoassay. The anti-nedocromil sodium antiserum solution was prepared by the addition of sheep anti-nedocromil antiserum (22 μl) and control sheep serum (200 μl) to the radioimmunoassay buffer (200 ml), or multiples of these volumes. The radioligand solution was prepared by the addition of reconstituted donkey anti-sheep IgG antiserum (5.0 ml) and stock radioligand solution (40 μl) to the radioimmunoassay buffer (195 ml), or multiples of these volumes. These solutions were prepared freshly each day and were stirred until used.

Preparation of radioimmunoassay tubes and subsequent manipulations. A Micromedic automated pipetting station with auxiliary pipette was used simultaneously to dispense

sample (100 μl) and to add anti-nedocromil sodium antiserum solution (0.5 ml) and radioligand solution (0.5 ml) to each radioimmunoassay tube. Standard plasma radioimmunoassay tubes were prepared in quadruplicate at the beginning of each analysis batch. Quality control plasma radioimmunoassay tubes, in duplicate or quadruplicate, were prepared immediately after preparation of the standard plasma radioimmunoassay tubes and also at the end of each analysis batch. Test plasma radioimmunoassay tubes were prepared in duplicate between preparation of the two sets of the quality control plasma tubes. When test urine samples, which were analysed as 20-fold, or greater dilutions (100 μl) in the radioimmunoassay buffer, were included in an analysis batch, buffer (100 μl) was added, using the second dispensing syringe of the auxiliary pipette, to the standard plasma and the quality control plasma tubes and also any test plasma tubes included in the analysis batch. The diluted test urine tubes also contained pooled blank human plasma (100 μl) which was added before, or at the same time as the test samples and the solutions.

During the development of the radioimmunoassay, and prior to the introduction of the use of the Micromedic automated pipetting station, samples were dispensed using an Eppendorf pipette (100 μl), and the antiserum and the radioligand solutions were dispensed via a Compu-pet dispenser.

The radioimmunoassay tubes were incubated overnight (16–20 h) at ambient temperature and then centrifuged (1900 **g**, 15 min) to deposit the antiserum-bound radioligand in the immune precipitates. The tubes were inverted over a sink to decant the supernatants and, maintained in an inverted position, were placed on a wad of tissues. The inverted tubes were transferred to a tray containing water to a depth of 1.0 cm and the procedure repeated using fresh water to remove any supernatant still adhering to the tubes. The bound radioligand was counted for 3 min so that at least 10,000 counts were accumulated for the nedocromil sodium-free standard plasma samples. The counts obtained from the standard plasma tubes were fitted to a four-parameter equation [8] using a radioimmunoassay programme (Program Version RIA — T2.1, copyright EMI Nuclear Enterprises Ltd.). The counts obtained for the other tubes were compared with the equation by the programme and the determined concentrations of nedocromil sodium in the samples were calculated from the results from the duplicate or quadruplicate tubes.

Results

Anti-nedocromil sodium antiserum production

After the first boosting immunisation, sera from all three sheep were found to bind [^{3}H]nedocromil sodium. After subsequent boosting immunisations, assessment of the sera on the basis of titre and also on the extent of displacement of bound [^{3}H]nedocromil sodium by a small amount of unlabelled nedocromil sodium indicated that the serum collected on day 69 from one of the sheep (reference 21-290382) was the most suitable antiserum for use in the method. The titre of this antiserum using the ^{125}I-radioligand and the secondary antiserum separation procedure was in the region of 1 in 20,000.

Properties of the radioimmunoassay

Standardisation and limit of detection. A typical standard curve is shown in Fig. 2. The binding of the radioligand in the nedocromil sodium-free standard plasma samples was 56% and the non-specific binding, estimated by the radioimmunoassay programme used

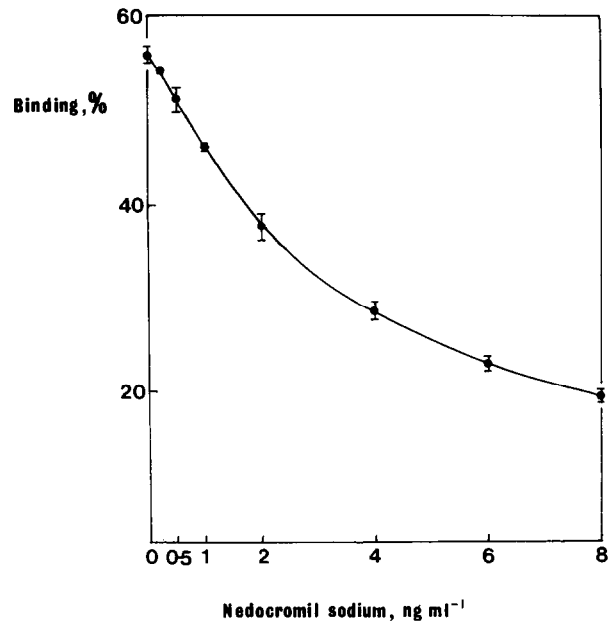


Figure 2
Standard curve for the determination of nedocromil sodium.

[8], was 6.6% in the illustrated example. The limit of detection of the radioimmunoassay, estimated according to the definition of Feldman and Rodbard [9] using twice the standard deviation of the binding responses for the nedocromil sodium-free standard plasma samples, was a concentration of less than 0.25 ng ml^{-1}. An estimate of the limit of detection was also obtained by the analysis of plasma samples collected periodically over 15 months from 17 donors, who were not receiving nedocromil sodium. Determination of the apparent nedocromil sodium concentrations in these blank plasma samples resulted in a mean of 0.011 ± 0.097 ng ml^{-1} (N = 49) from which, applying the above criteria of two standard deviations, the limit of detection was estimated to be 0.21 ng ml^{-1}. A concentration of nedocromil sodium of 0.25 ng ml^{-1} is therefore an appropriate limit of detection for the analysis of plasma samples.

Since urine samples are routinely analysed after a 20-fold dilution, the limit of detection of the radioimmunoassay for the analysis of urine samples, based on the limit for plasma samples and assuming no contribution to the magnitude of the limit by the urine, may be calculated to be in the region of 5 ng ml^{-1}. Analysis of blank 24 h collection urine samples from 24 donors resulted in an experimentally estimated limit of detection of 6 ng ml^{-1}, in good agreement with the above calculated value.

Accuracy and precision. The intra- and inter-assay mean accuracy and precision of the method were determined by the repetitive analysis of quality control plasma samples (Table 1). The inter-assay relative standard deviation of the method was less than 6.0% at the two higher concentrations of nedocromil sodium studied (2.0 and 6.0 ng ml^{-1}) and was 15.1% at 0.5 ng ml^{-1} which is a concentration twice the limit of detection of the method.

Table 1
Intra-assay and inter-assay accuracy and precision of the method

Concentration of nedocromil sodium ng ml^{-1}	Intra-assay Accuracy %	Intra-assay Relative standard deviation %	Intra-assay Number of duplicate radioimmunoassay tubes analysed	Inter-assay Accuracy %	Inter-assay Relative standard deviation %	Inter-assay Number of assays in which duplicate radioimmunoassay tubes were analysed
0.5	112.4	7.8	16	107.2	15.1	18
2.0	102.7	3.9	16	103.3	5.0	18
6.0	102.2	4.4	16	101.5	5.6	18

Specificity with respect to cross-reaction of drugs with the anti-nedocromil sodium antiserum. As a measure of the specificity of the radioimmunoassay, the extent of cross-reaction with the anti-nedocromil sodium antiserum of a range of drugs and compounds (Table 2) was determined by the method of Abraham [10]. Most of the drugs and compounds were studied at a maximum concentration of 5 $\mu g\ ml^{-1}$. Certain of the drugs (sodium salicylate, sodium acetylsalicylate and phenylbutazone) were studied at maximum concentrations of 250 $\mu g\ ml^{-1}$ since the therapeutic concentrations of these drugs can be much greater than 5 $\mu g\ ml^{-1}$. None of the drugs, except the closely related ambicromil sodium, cross-reacted significantly (Table 2) with the anti-nedocromil sodium antiserum. With the exception of ambicromil sodium therefore, the radio-immunoassay is free of interference by the listed drugs.

Table 2
Specificity of the anti-nedocromil sodium antiserum (21-290382)

Compound	Cross-reaction relative to nedocromil sodium %
Nedocromil sodium	100.00
Ambicromil sodium (FPL 52370)*	6.00
Minocromil (FPL 59360)†	0.06
Sodium cromoglycate‡	
Imipramine hydrochloride	
Theophylline	
Warfarin	
Chlorpromazine hydrochloride	
Sodium prednisolone-21-succinate	<0.04
Terbutaline sulphate	
Isoprenaline sulphate	
Salbutamol sulphate	
Beclomethasone dipropionate	
Phenylbutazone	
Sodium salicylate	<0.001
Sodium acetylsalicylate	

*Disodium 4,6-dioxo-10-propyl-4H,6H-benzo[1,2-b:5,4-b']dipyran-2,8-dicarboxylate.
†6-Methylamino-4-oxo-10-propyl-4H-pyrano[3,2-g]quinoline-2,8-dicarboxylic acid.
‡Disodium 5,5'[(2-hydroxytrimethylene)dioxy]bis[4-oxo-4H-1-benzopyran-2-carboxylate].

Comparison of the radioimmunoassay with liquid scintillation counting for the determination of [^{14}C]nedocromil sodium in plasma and urine samples. Two subjects received [^{14}C]nedocromil sodium by slow intravenous infusion and plasma and urine samples were collected. The concentrations of [^{14}C]nedocromil sodium in the samples were determined both by the radioimmunoassay, after dilution, where appropriate, and by liquid scintillation counting. Since subsequent analysis of the ^{14}C-labelled material in the samples by high-performance liquid chromatography demonstrated that at least 98% of the radioactivity co-chromatographed with nedocromil sodium, the liquid scintillation determinations constituted valid measures of [^{14}C]nedocromil sodium. Comparisons of the plasma and urine concentrations of [^{14}C]nedocromil sodium determined by the radioimmunoassay with those determined by liquid scintillation counting are illustrated in Figs 3 and 4 respectively. Logarithmic axes are used in Fig. 4 since a wide range of concentrations of nedocromil sodium were present in the urine samples. The regression equations for both the plasma and urine sample results had slopes very close to unity.

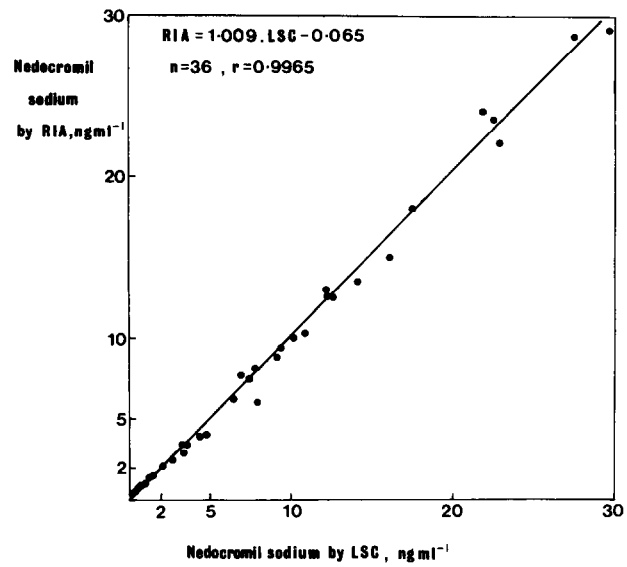


Figure 3
Comparison of concentrations of nedocromil sodium determined by the radioimmunoassay in plasma samples from subjects receiving [^{14}C]nedocromil sodium with concentrations determined by liquid scintillation counting.

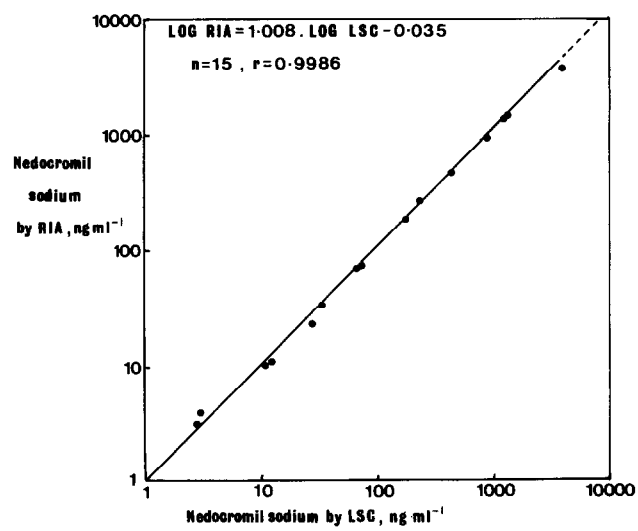


Figure 4
Comparison of concentrations of nedocromil sodium determined by the radioimmunoassay in urine samples from subjects receiving [^{14}C]nedocromil sodium with concentrations determined by liquid scintillation counting.

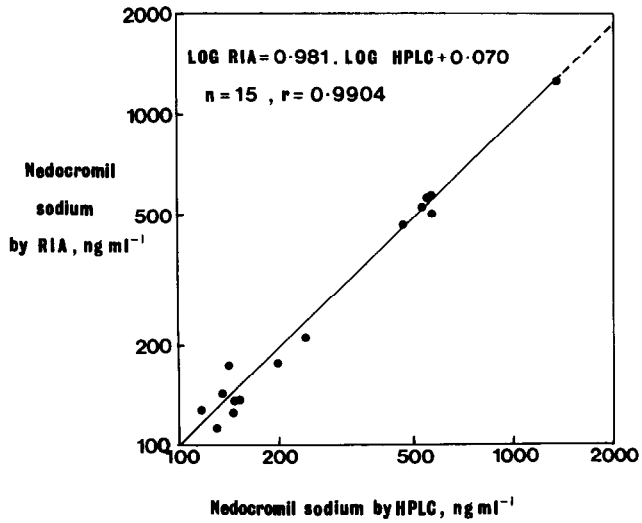


Figure 5
Comparison of concentrations of nedocromil sodium determined by the radioimmunoassay in urine samples from subjects receiving nedocromil sodium (4 mg) from a pressurised aerosol (Tilade®) with concentrations determined by a high-performance liquid chromatographic method.

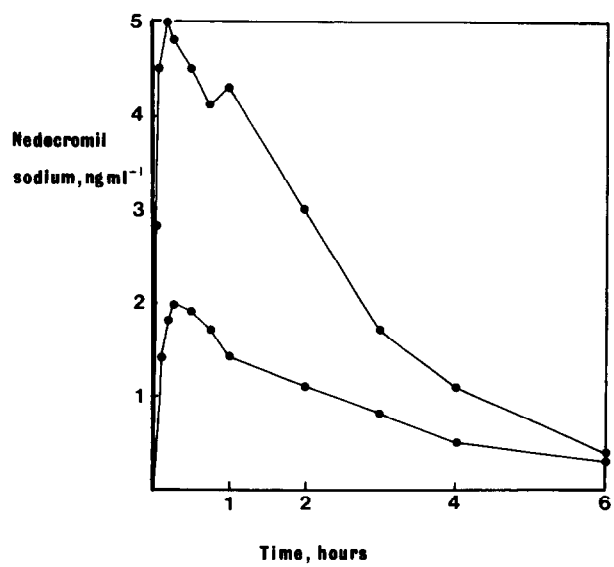


Figure 6
Plasma concentrations of nedocromil sodium found in two subjects who received 4 mg of the compound from a pressurised aerosol (Tilade®).

Comparison of the radioimmunoassay with a high-performance liquid chromatographic method for the determination of nedocromil sodium in urine samples. Urine samples from six subjects who received nedocromil sodium (4 mg) by inhalation from a pressurised aerosol were analysed both by the radioimmunoassay and by a high-performance liquid chromatographic method, which involved solvent extraction, reversed-phase chromatography and ultraviolet absorbance detection. Due to the relative insensitivity of the chromatographic method, results were obtained by both methods for the urine samples collected in the periods 0–2 and 2–4 h for all the subjects and in the period 4–6 h for three of the subjects only. Comparison of the results obtained (Fig. 5) indicated good agreement between the two analysis methods.

Applicability of the radioimmunoassay to the analysis of plasma and urine samples from subjects who had received a single dose of nedocromil sodium by inhalation from a pressurised aerosol. The radioimmunoassay was used to determine the concentrations of nedocromil sodium in plasma samples taken from two subjects who had each received a single nominal dose of 4 mg of the compound by inhalation from a pressurised aerosol. The plasma concentration versus time curves obtained are illustrated in Fig. 6. In these two subjects, and the majority of other subjects and patients studied, concentrations of nedocromil sodium in the plasma remained greater than the limit of detection of the radioimmunoassay (0.25 ng ml^{-1}) for up to 6, or even 8 h, after the administration of the compound. Concentrations determined by the radioimmunoassay in urine samples from the same two subjects varied from a maximum concentration of 2800 ng ml^{-1}, in the 0–1 h sample from one of the subjects, to 18 and 16 ng ml^{-1}, in the 12–24 h samples.

Discussion

Since nedocromil sodium is not metabolised, the preparation of the anti-nedocromil sodium antisera did not require minimisation of metabolite cross-reactivity [11]. Instead, antisera were raised in sheep against an easily prepared heterogeneous immunogen, a mixture of compounds III and IV (Fig. 1). Suitable antisera against the immunogen were subsequently selected by titre and by a simple test of potential assay sensitivity [12]. In this test, the ease of displacement of bound radioligand by a fixed small amount of unlabelled nedocromil sodium was measured. Although selected on the above basis, the final radioimmunoassay, developed using antiserum 21-290382 in combination with a radioligand heterogeneously labelled with iodine-125, is highly specific. Negligible cross-reaction of the antiserum was found (Table 2) with a wide range of drugs, including minocromil, which have some structural resemblance to nedocromil sodium. Ambicromil sodium which is a closer structural analogue of nedocromil sodium was found to exhibit a small degree of cross-reaction. However, even in this case the cross-reaction was only 6.0%. Additionally, the results obtained by the radioimmunoassay for both plasma and urine samples agree well with the results obtained by other analysis methods, providing further support for the specificity of the method.

The method has proved suitable for the determination of nedocromil sodium in plasma samples from volunteers or patients up to 6 or 8 h after the administration of the compound by inhalation. However, with some pre-dose plasma samples, from subjects in whom the patency of cannulae used for blood sample collection was maintained using heparinised saline, a negative interference with the radioimmunoassay, which rarely exceeded 1.0 ng ml^{-1}, was seen. A similar interference, due to an *in vivo* effect of

heparin, has been described for other radioimmunoassays [13, 14]. Hence, when blood is collected for determination of the plasma concentration of nedocromil sodium by the radioimmunoassay, the use of heparinised saline to maintain the potency of cannulae should be avoided. Heparin used *in vitro* to anticoagulate collected blood does not produce the interference. Further investigation of the mechanism of the *in vivo* heparin effect is being carried out.

In common with many other radioimmunoassays the nedocromil sodium assay employs a secondary antibody to separate bound and free radioligand. However, in the nedocromil sodium assay the customary separate primary and secondary immune incubation periods are replaced by a single overnight incubation; the primary antiserum, radioligand and secondary antiserum being added simultaneously to the radioimmunoassay tubes. This procedure, in conjunction with the use of automated equipment for the preparation of the radioimmunoassay tubes, has resulted in a method which is experimentally simple and robust, and which may be used for the analysis of large numbers of samples in a single batch. Indeed, the radioimmunoassay has been extensively used over a period of more than two years for the analysis of plasma and urine samples generated in pharmacokinetic studies on volunteers and patients.

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