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Short communication

Validation of a high-performance liquid chromatography assay for urinary nedocromil sodium following oral and inhaled administration

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Abstract

The validation of a solid-phase extraction and an ion pair high-performance liquid chromatographic assay for the determination of nedocromil sodium (NCS) in urine samples following oral and inhaled administration to healthy volunteers is described. NCS and its internal standard sodium cromoglycate (SCG) were extracted from urine samples using solid-phase extraction and then quantified using high-performance liquid chromatography (HPLC). A 25-cm C_8 Spherisorb 5 μm stationary phase with a mobile phase containing a long alkyl chain ion-pair reagent (methanol–0.045 *M* phosphate buffer–0.05 *M* dodecyl triethyl ammonium phosphate; 550:447.6:2.4, v/v) was used. The mean (S.D.) intra-day accuracy and precision of the HPLC assay was 99.9 (1.6) and 7.05 (4.9)%, respectively. These values for the inter-day data were 102.4 (4.07) and 10.5 (2.7)%, respectively, over the concentration range investigated. The method described permits the detection of NCS in human urine at concentrations as low as 0.04 μg ml^{-1} where the signal-to-noise ratio is greater than 3:1. In 10 healthy volunteers a significantly greater amount of NCS was excreted in the urine following inhalation than after oral dosing ($p<0.001$). The mean (S.D.) amount of NCS renally excreted at 0.5, 1.0 and 24 h following inhalation of four 2-mg doses of NCS from a metered dose inhaler (MDI) was 0.513 (0.24), 1.163 (0.49) and 4.00 (1.73)% of the nominal dose. Similar values after oral administration of 8 mg of NCS were 0.026 (0.03), 0.079 (0.06) and 0.930 (0.74)%, respectively.

Keywords: Nedocromil sodium

1. Introduction

Nedocromil sodium (NCS) is the disodium pyranoquinoline dicarboxylate, used for the treatment of reversible bronchoconstrictive diseases [1,2]. Like all drugs delivered by metered dose inhalers (MDIs) only a small proportion of the actuated NCS dose reaches the lungs following inhalation. The majority of the dose is deposited in the oropharyngeal region and is subsequently swallowed. Although NCS is well absorbed from the lungs, only 2–3% of the oral dose is absorbed from the gastrointestinal tract [3]. Thus only small amounts of NCS (less than 10%) are delivered into the systemic circulation. Pharmacokinetic methods to assess the pulmonary deposition of NCS are, therefore, difficult due to the problems associated with the assay of low serum or plasma drug concentrations.

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Hindle and Chrystyn [4] reported a urinary excretion method for assessing the relative lung bioavailability of inhaled salbutamol. They demonstrated that after oral administration of salbutamol, negligible amounts of salbutamol are excreted 30 min post dose while significantly greater amount are excreted 30 min post inhalation. Also similar amounts were excreted over the 24 h period post oral and inhaled dosing. Therefore the amount of the drug in the urine sample taken 30 min post inhalation is representative of the amount of drug delivered to the lungs (relative bioavailability of salbutamol to the lungs following inhalation) while the 24 h recovery reflects the total systemic availability (relative bioavailability of the salbutamol to the body following inhalation).

NCS is eliminated unchanged, from the systemic circulation, by the renal route [5]. In order to evaluate a similar urinary excretion method, to that of salbutamol [4], for inhaled NCS, a sensitive analytical method is required. A radioimmunoassay using a mono-tyramine derivative of NCS labelled with iodine-125 has been used for the quantification of NCS in plasma and urine at ng ml^{-1} levels [6]. Despite its good specificity and sensitivity, this method is costly and tedious for routine clinical studies. A high-performance liquid chromatography (HPLC) method for the determination of NCS from urine has been reported by Baker et al. [7]. Following direct injection of urine this method uses a reversed-phase column to concentrate the analyte. The analyte is then automatically back-washed from the concentration column onto an ion-exchange column where final separation of NCS from urine constituents occurs. When automated by computer control this method is simple to perform and manual pre-treatment of urine samples is not necessary. However, the direct injection of urine, a large injection volume, a high buffer concentration, a very high flow-rate and the number and length of the columns used, will cause a very high operating back-pressure and short column life.

We have previously reported a reversed-phase ion-pair HPLC assay for SCG in the urine which used NCS as the internal standard [8]. Analysis of urine samples post SCG inhalation revealed the potential for this method to determine the relative bioavailability of SCG to the lung following inhalation. We report here the validation of this HPLC assay and the urinary excretion profile of NCS following oral and inhaled administration.

2. Experimental

2.1. Chemicals and standard solutions

Tilade® 2 mg metered dose inhaler, nedocromil sodium and sodium cromoglycate were gifts from Rhone Poulenc Rorer (Loughborough, UK). The water content of NCS and SCG was determined, using Karl-Fischer titration, immediately prior to the preparation of aqueous standards. This enabled accurate standard solutions to be prepared because NCS and SCG readily absorb water on storage. Dodecyl triethyl ammonium phosphate (HPLC grade) was supplied by Regis (IL, USA). All other chemicals were purchased from BDH (Poole, UK) and were of analytical or HPLC grade (as appropriate). Highly purified double distilled water was used throughout the study.

Aqueous stock solutions of NCS and SCG 200 mg l^{-1} (w/v) were prepared every two months. These stock solutions were stored at room temperature and showed good stability over that period of time. From the NCS stock solution, working standards were prepared by serial dilution with pooled 24 h urine collected from three (one female) volunteers to yield nominal NCS concentrations of 0.075, 0.15, 0.3, 0.75, 1.5 and 3.0 mg l^{-1}. Working solutions were stored below -20°C prior to analysis.

2.2. Instrumentation and chromatographic conditions

The HPLC system and solid-phase extraction method using phenyl (PH) Bond Elut (Varian, CA, USA) cartridges, which was used to isolate SCG and NCS from urine has been previously reported [8].

Phenyl (PH) cartridge were conditioned with 2 ml methanol followed with 2 ml 0.1 *M* hydrochloric acid. To 2 ml of urine was added 8 ml of 0.1 *M* hydrochloric acid containing the internal standard, this was adjusted (if necessary) to pH 1 and then applied to the cartridges over 2 to 3 min. After applying a full vacuum for 5 min, the cartridges were washed with 2 ml of 0.1 *M* hydrochloric acid,

followed by 2 ml of 32% (v/v) methanol in 0.1 *M* hydrochloric acid. Cartridges were dried again using a full vacuum for another 5 min and the analyte was then eluted with 2 ml methanol and collected in sample vials. After evaporation to dryness using a nitrogen stream, the samples were reconstituted in 1 ml mobile phase and 20 μl was injected into the HPLC system. The HPLC stationary phase was a 25 cm×4.6 mm I.D. C_8 Spherisorb 5 μm column (Hichrom, Reading, UK) with a 1 cm×4.6 mm I.D. guard column containing the same material. The mobile phase was methanol–0.045 *M* phosphate buffer–0.5 *M* dodecyl triethyl ammonium phosphate (550:447.6:2.4, v/v) adjusted to pH 2.3 by the addition of concentrated orthophosphoric acid. It was filtered through a 0.45-μm membrane filter (Millipore) and degassed under vacuum in an ultrasonic bath for 30 min prior to use. A constant flow-rate of 0.85 ml min^{-1} was used and UV detection was set at 256 nm. Ambient temperatures were used throughout the analysis.

2.3. Volunteer study

Local Ethics Committee approval was obtained for 10 healthy volunteers (five female) whose mean (S.D.) age, weight and height was 32.5 (8.3) years, 72.18 (11.3) kg and 173.4 (7.9) cm, respectively. All volunteers were trained in a metered dose inhalation technique prior to inclusion, and gave their written informed consent. On study days subjects either inhaled four doses from a Tilade® (2 mg NCS per dose) metered dose inhaler (MDI) or swallowed 8 mg NCS powder dissolved in 25 ml of water. The order of administration was randomised, and there was a seven-day period between the study days. Each inhaled dose was separated by 30 s and thus to compensate for this the 8 mg oral dose was administered as four aliquots of 2 mg swallowed every 30 s. Immediately prior to each study dose, subjects emptied their bladder. Urine was then collected at 0.0, 0.5, 1.0, 2.0, 5.0, 8.0, 12, 24 and 36 h post-dose. All samples were frozen at −20°C prior to extraction and HPLC analysis. Stability studies over nine months of storage at −20°C and through three thawing cycles showed no significant change in analyte concentration.

3. Results

3.1. Chromatography

Chromatograms obtained from the analysis of an extracted blank urine sample, a standard urine sample containing 2 μg ml^{-1} NCS and 1 μg ml^{-1} SCG, together with a volunteer sample 0.0–0.5 h post-inhalation and post-oral administration are presented in Fig. 1a, 1b, 1c, and 1d, respectively.

NCS and SCG eluted from the column with capacity factors (k) of 13.3 and 8.5, and retention times of 43.1 and 28.7 min, respectively. The baseline resolution factor of NCS:SCG peaks was 26.2±0.3 (mean±S.D., n=8).

3.2. Method validation

The calibration curve was linear over the concentration range of 0.075 to 3.0 μg ml^{-1} NCS in urine, with regression coefficients >0.9997 and the intercept did not differ significantly from zero (a< 0.001). The peak height of the urine standards after extraction was compared to the peak height of aqueous NCS standards after direct injection [9]. Four extractions from urine containing NCS concentrations of 0.075, 0.15, 0.75, 1.5 and 3.0 μg ml^{-1} provided recoveries of 82.5, 90.5, 90.6, 91.5, 95.7 and 91.6%, respectively. The mean (S.D.) absolute recovery of NCS from urine, using this method, was 90.4±4.3%.

The accuracy and the intra- and inter-day precision (mean percentage coefficient of variation) of the assay are presented in Table 1. The mean (S.D.) intra-day assay variability, determined for the six standard urine concentrations of NCS on four occasions, was 7.05 (4.9)%. Inter-day assay variability, determined at the same six concentrations using four replicate runs on different days, was 10.5 (2.7)%. The accuracy of the assay, calculated from comparison of the nominal NCS concentration to the actual concentration obtained from the linear regression line was <10% over the concentration range investigated (0.075 to 3.0 μg ml^{-1}).

The limit of detection (LOD) and the limit of quantitation (LOQ), were calculated from the mean and S.D. of the intercept of four calibration curves. A value of greater than 3:1 for the signal-to-noise ratio

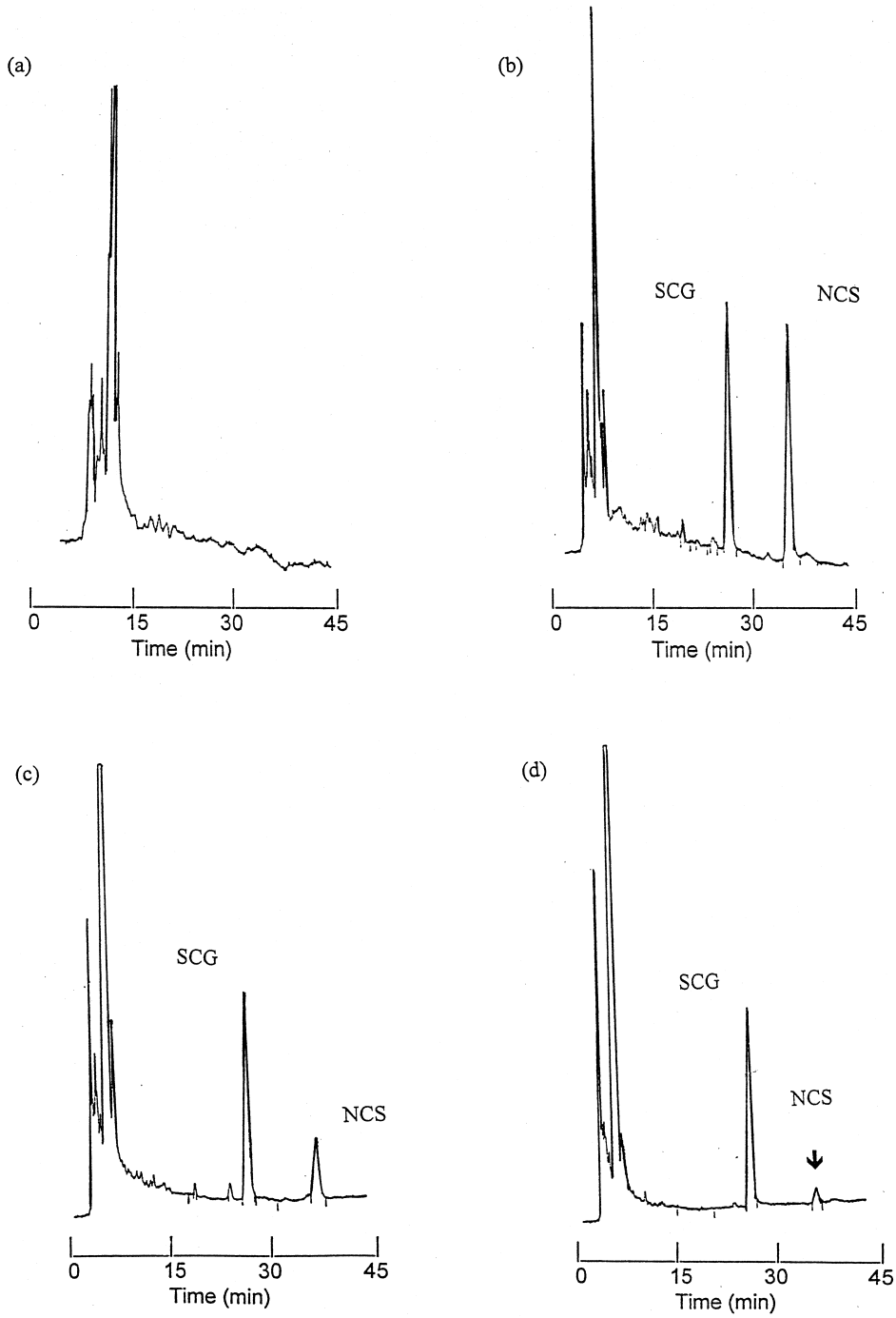

Fig. 1. Chromatograms of (a) blank urine sample, (b) a standard urine sample containing 2 μg ml^{-1} SCG and 1.5 μg ml^{-1} NCS, (c) a volunteer urine sample 0–0.5 h post inhalation containing 0.4 μg ml^{-1} NCS and (d) the same volunteers urine sample 0–0.5 h post oral dose containing 0.08 μg ml^{-1} NCS.

Table 1
Precision and accuracy of the nedocromil sodium HPLC assay

Nominal NCS concentration ($\mu g\ ml^{-1}$)	Mean (S.D.) $\mu g\ ml^{-1}$ of measured NCS concentration ($n=4$)	Difference between nominal and found concentration (%)	Coefficient of variation (%)
Intra-assay variation			
0.075	0.073±0.01	2.7	12.16
0.15	0.151±0.02	0.7	14.33
0.30	0.304±0.01	1.5	3.94
0.75	0.757±0.02	1.0	3.13
1.50	1.484±0.09	1.1	5.78
3.00	3.000±0.09	0.0	2.94
Inter-assay variation			
0.075	0.081±0.01	8.6	14.60
0.15	0.158±0.02	5.7	11.33
0.30	0.304±0.03	1.6	8.26
0.75	0.752±0.09	0.3	12.23
1.50	1.459±0.11	2.8	7.65
3.00	3.030±0.27	1.1	9.12

was used for the LOD and of greater than 10:1 for the LOQ. Under procedural conditions the LOD and LOQ using a 1-ml urine sample and a 20-μl injection volume were 0.04 $\mu g\ ml^{-1}$ and 0.075 $\mu g\ ml^{-1}$, respectively.

To determine the specificity of the assay a 24 h drug-free urine collection from seven volunteers (three female) was spiked with drugs commonly used in the management of asthma. The extracted spiked urine was injected to the HPLC system and no interfering peaks were observed.

The cumulative amount of NCS excreted in the urine post inhaled and oral dosing is shown in Fig. 2. Significantly more NCS was excreted in the 0.5, 1.0 and 24 h following inhalation compared to oral administration ($p<0.001$). The mean (S.D.) cumulative amounts of NCS excreted at 0.5, 1.0 and 24 h post inhaled and oral dosing, as shown in Fig. 3 were

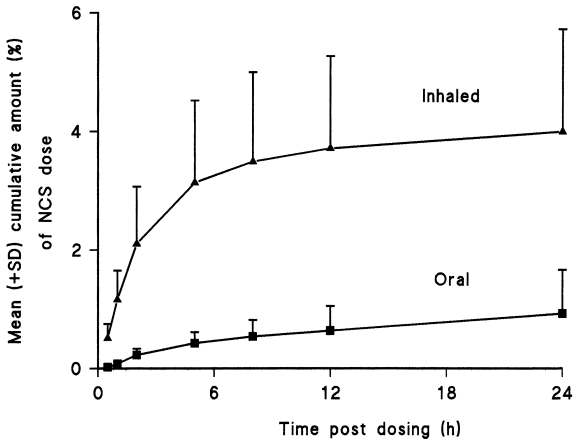

Fig. 2. Mean (S.D.) % cumulative amount of NCS excreted in the urine post inhaled and oral dosing.

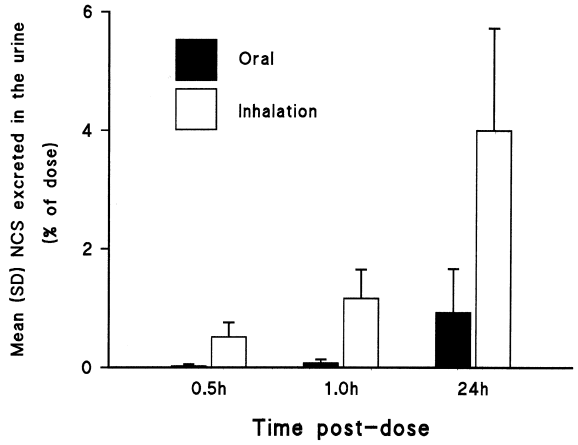

Fig. 3. Mean (S.D.) cumulative amount of NCS excreted at 0.5, 1.0 and 24 h post dosing.

0.513 (0.24), 1.163 (0.49) and 4.00 (1.73)% and 0.026 (0.03), 0.079 (0.06) and 0.930 (0.74)%, respectively.

4. Discussion and conclusions

The reversed-phase ion-pair HPLC assay described by Aswania et al. [8], for the determination of urinary SCG may also be used to determine the concentration of NCS in urine samples post dose. The clean-up stage using PH solid-phase cartridges isolated NCS and SCG from urinary endogenous substances that might interfere with the assay and gave a highly, reproducible absolute recovery of both compounds (>90%). The addition of an alkyl triethyl ammonium phosphate ion-pair reagent to the mobile phase enhanced the baseline resolution of the NCS and SCG from other interfering compounds. The assay has acceptable limits for both accuracy and precision and has been successfully used to analyse samples from this study, and other subsequent clinical studies. Compared with other published methods [6,7], this assay is simple, sensitive (LOD is 0.04 $\mu g\ ml^{-1}$) and suitable for routine clinical studies. With automatic injection up to 20 samples can be analysed in one day.

The urinary excretion of NCS in the first 30 min post inhalation when compared to that following oral dosing indicates that this method could be used to compare the relative lung deposition of two inhaled nedocromil products or methods. Thus a similar technique to that reported for salbutamol [4], could be used for nedocromil sodium.

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