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Journal of Chromatography B, 668 (1995) 59–65

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Automated high-performance liquid chromatographic method for the determination of nedocromil sodium in human urine using bimodal column switching

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First received 2 June 1994; revised manuscript received 24 January 1995; accepted 24 January 1995

Abstract

An automated HPLC method is described for the determination of nedocromil sodium in human urine. An HPLC autosampler is used to inject urine samples onto a short reversed-phase column. This column acts as a concentration column and performs a preliminary extraction. The concentration column is automatically back-flushed onto an ion-exchange column where final separation of nedocromil sodium from urine constituents occurs. Recovery, accuracy, precision, sensitivity and specificity were investigated. The method has been applied to urine samples from clinical studies, and the results were compared to those obtained using a radioimmunoassay developed previously.

1. Introduction

Nedocromil sodium (disodium 9-ethyl-6,9-dihydro-4,6-dioxo-10-propyl-4H-pyrano-[3,2-g]-quinoline-2,8-dicarboxylate; Fig. 1) is a compound possessing anti-inflammatory properties suitable for treating asthma and other reversible obstructive airways diseases. A method was required to support the development of the compound during clinical trials for conditions such as reversible obstructive airways disease [1,2] during which the compound is inhaled from a pressurised aerosol (Tilade) into the lungs.

A radioimmunoassay has been developed for the determination of the drug in plasma and urine [3]. The use of the radioimmunoassay involves tedious manipulations and the method has a limited range requiring urine samples to be diluted several times before analysis. To provide an alternative and improved way of determining

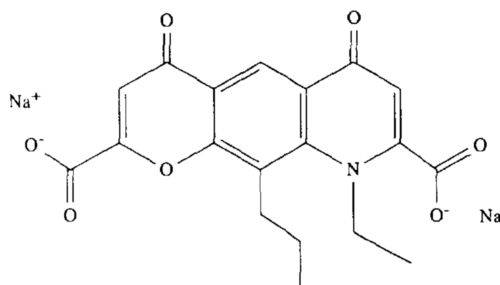

Fig. 1. Structure of nedocromil sodium.

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SSDI 0378-4347(95)00049-6

nedocromil sodium in urine HPLC methods have been examined. An HPLC method following solvent extraction was investigated, unfortunately a lack of specificity restricted the lower limit of quantitation to approximately 0.1 μg/ml. Since column switching has previously been used to automate biological sample extraction and analysis [4], this approach was investigated for nedocromil sodium.

Effective column-switching methodology is facilitated by the use of two different modes of separation. Ion-exchange chromatography has previously been used for the determination of sodium cromoglycate, another chromone carboxylic acid [5]. Since ion-exchange and reversed-phase chromatography both use aqueous mobile phases there should be no serious compatibility differences. These modes of separation were therefore chosen to develop a method which should have good selectivity and specificity.

The method described uses an automated column-switching technique to extract and analyse urine samples. No manual pretreatment of urine samples is required. Urine is injected directly onto a short reversed-phase column, this is then washed with a mixture of methanol and diluted sulphuric acid to remove salts and other compounds significantly more polar than nedocromil sodium. The short column is then back flushed onto an ion-exchange column, where final separation of nedocromil sodium from urine constituents takes place.

2. Experimental

2.1. Chromatographic system

The chromatographic system is illustrated in Fig. 2. A Waters Model M45 pump (Waters Chromatography Division, Millipore Corporation, Milford, MA, USA) was used to deliver mobile phase to the concentration column and a Waters Model 510 pump was used to deliver mobile phase to the analytical column. Both pumps were controlled by a Waters Model 720 system controller. A Perkin-Elmer ISS-100 autosampler (Perkin-Elmer Corporation, Norwalk, CT, USA) fitted with a Rheodyne valve and a preparative sample loop (2.0 ml) was used to inject urine samples. All columns were housed within a Perkin-Elmer LC-100 column oven. A Knauer automatic six-port valve (Dr H Knauer, Berlin, Germany) was used for column switching and was controlled by the system controller, via a relay. The in-line filter was used to remove any particulate matter which was injected with the

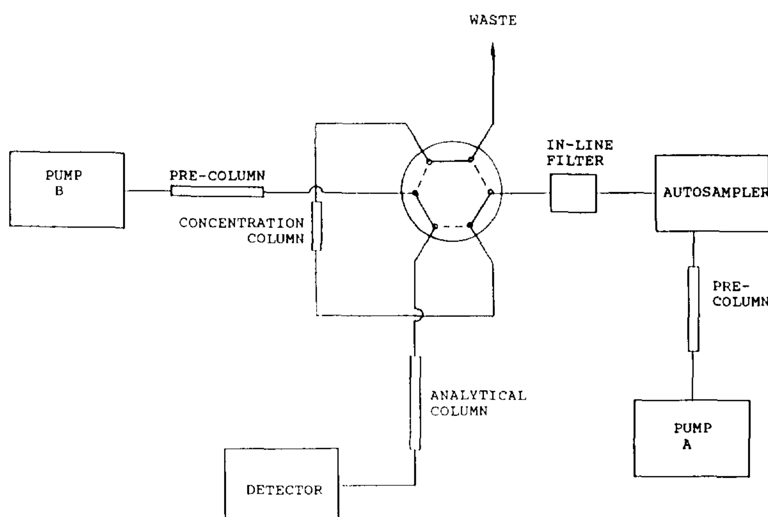

Fig. 2. Configuration of the HPLC system.

samples. A Schoeffel Model SF 769 spectroflow monitor (Spectros, Manchester, UK) was used to monitor at 253 nm the effluent from the analytical column. A Hewlett-Packard 5880A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a second terminal and an external analogue input board, was used as an integrator. On some occasions a Spectra-Physics SP4270 integrator (Spectra-Physics, San Jose, CA, USA) was used.

A pre-column was placed after each pump to saturate the mobile phases with silica and to prevent impurities in the mobile phases being concentrated on the concentration column. The pre-columns were constructed by dry packing old analytical columns (250 × 4.6 mm I.D.) with silica (25–40 μm, HPLC Technology, Macclesfield, UK). The concentration column consisted of a Knauer cartridge column (30 × 4.0 mm I.D.) filled with Hypersil 5-ODS. The analytical column was a Zorbax SAX column (250 × 4.6 mm I.D., Du Pont, Wilmington, DE, USA).

2.2. Chemicals

All reagents were of analytical reagent grade (FSA Laboratory Supplies, Loughborough, UK).

2.3. Standard curves

Standard solutions of nedocromil sodium (2 and 100 μg/ml) in water were added to samples of pooled blank urine to obtain standard urine samples at concentrations of 0, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.5, 5 and 10 μg/ml. The standard urine samples were analysed using the same procedure as for test urine samples. A standard curve was constructed by plotting the logarithm of the peak heights, reported by the integrator, against the logarithm of the concentration of each standard sample.

2.4. Mobile phases

Mobile phase A was prepared by mixing sulphuric acid (0.092 mol/l) with methanol in the ratio 70:30. Phosphate buffer was prepared by mixing disodium hydrogen orthophosphate (0.18 mol/l) with orthophosphoric acid (0.18 mol/l) to achieve a pH of 3.00 ± 0.05. Mobile phase B was prepared by mixing the phosphate buffer with an equal volume of methanol.

Mobile phases were filtered through cellulose nitrate membrane filters (0.45 μm) and degassed with helium before use.

2.5. Chromatographic procedure

Urine samples (1 ml) were pipetted into autosampler vials. Injections (200 μl) were made by the autosampler onto the concentration column, with mobile phase A at a flow-rate of 5 ml/min. After 1.5 min the six-port valve was switched and the flow-rate of pump A was reduced to 0.2 ml/min. Pump B back-flushed the sample, from the concentration column, onto the analytical column with mobile phase B at a flow-rate of 1 ml/min. The six-port valve was returned to the original position 5.5 min after sample injection. Pump B continued to elute the analytical column with mobile phase B at a flow-rate of 1 ml/min.

3. Results and discussion

The eluent from the extraction column could be monitored by connecting a detector to the waste outlet from the switching valve. The methanol concentration of mobile phase A was adjusted so that the retention time of nedocromil sodium on the extraction column was approximately 3.0 min. This ensured that when the eluent was switched to the analytical column, nedocromil sodium had eluted approximately half way down the extraction column. The addition of methanol to this mobile phase produces a cleaner extract than sulphuric acid alone.

Typical chromatograms of urine samples are shown in Fig. 3. Standard curves were calculated for eight analysis batches. The shape of the standard curves and the correlation coefficients are listed in Table 1. Two of the curves were linear, but six exhibited a slight decrease in slope above concentrations of approximately 2.5 μg/ml. These non-linear curves were constructed

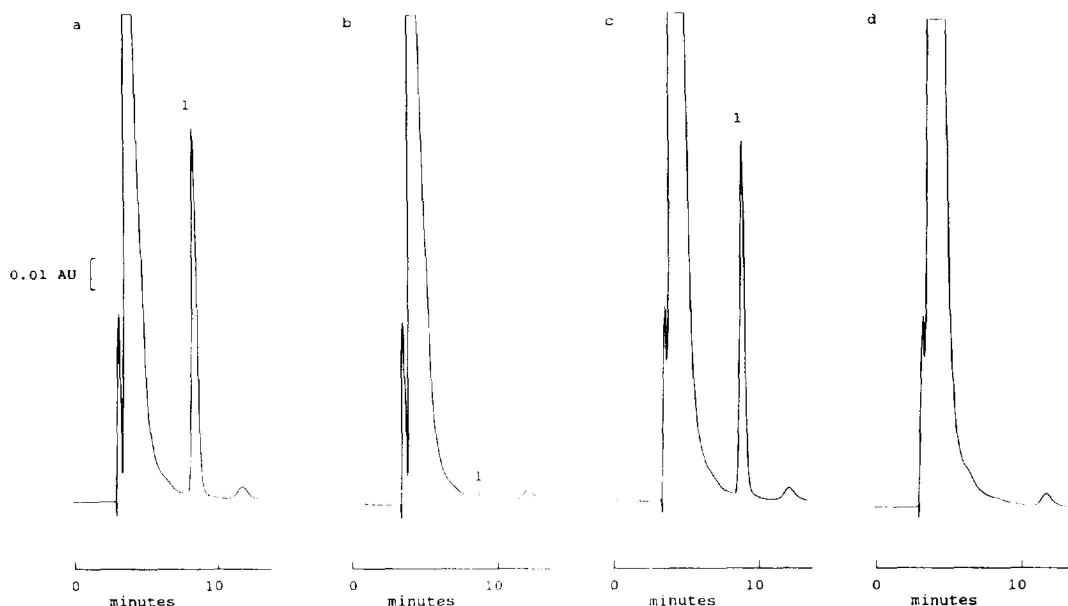

Fig. 3. Chromatograms of nedocromil sodium in urine samples. (a) Standard sample containing 2.5 μg/ml of nedocromil sodium. (b) Standard sample containing 0.02 μg/ml of nedocromil sodium. (c) Sample from a patient containing 2.41 μg/ml of nedocromil sodium. (d) Sample from a patient containing no detectable nedocromil sodium.

using a quadratic equation, with a negative quadratic term. The non-linearity was assumed to be due to the concentration range of the analyte exceeding the linear range of the detector.

Recovery was determined by injecting an aqueous solution of nedocromil sodium (10 μg/ml) directly onto the analytical column and comparing the size of the peak produced to the size of peaks produced from standard urine samples, analysed using the column-switching procedure. The recovery of nedocromil sodium, from urine, was 109%. The high recovery is probably due to the different injection techniques producing differently shaped peaks. These are shown in Fig. 4.

Table 1
Standard curve data for determination of nedocromil sodium in urine

Analysis batch	Shape of standard curve	Correlation coefficient of standard curve
1	Quadratic	0.99998
2	Quadratic	0.99996
3	Quadratic	0.99996
4	Linear	0.99992
5	Linear	0.99990
6	Quadratic	0.99987
7	Quadratic	0.99979
8	Quadratic	0.99975

The accuracy and precision of the method were investigated by replicate analysis of pooled urine samples containing added concentrations of nedocromil sodium. The intra-day accuracy and precision data are presented in Table 2 and the inter-day accuracy and precision data are presented in Table 3. The intra-day and inter-day coefficients of variation for the sample at a concentration of 0.01 μg/ml were considered unacceptable, at over 20%. The limit of quantitation was therefore considered to be 0.02 μg/ml, where the intra-day and inter-day coefficients of variation were less than 10%.

The specificity of the method, with respect to interference by urine constituents, was investigated by analysing urine from eight volunteers.

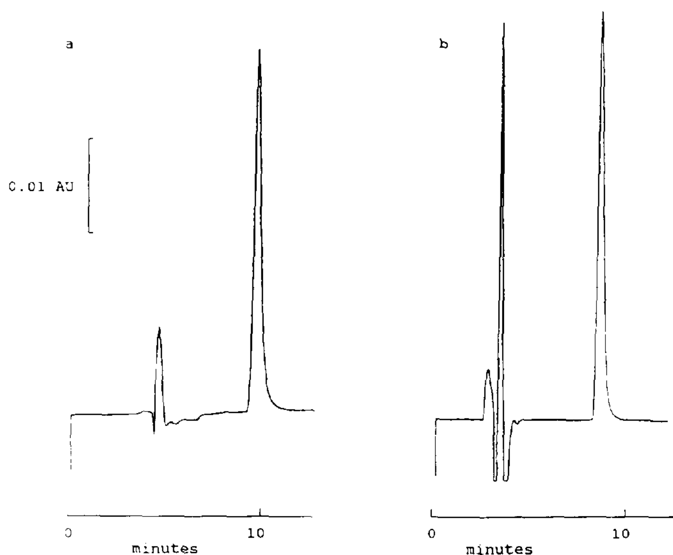

Fig. 4. Chromatograms of nedocromil sodium. (a) From an aqueous sample of nedocromil sodium (10 μg/ml) injected directly onto the analytical column. (b) From a standard urine sample containing nedocromil sodium (10 μg/ml), after column switching onto the analytical column.

Table 2
Intra-day accuracy and precision data

Concentration added (μg/ml)	Mean accuracy (%)	C.V. (%)	n
0.01	90	22	10
0.02	100	6.0	10
0.25	101	0.5	9
0.5	100	1.3	10
10	97	0.8	10

Table 3
Inter-day accuracy and precision data

Concentration added (μg/ml)	Mean accuracy (%)	C.V. (%)	n
0.01	100	21	6
0.02	105	8.1	7
0.25	100	2.5	7
0.5	100	2.0	7
10	98	2.2	8

The urine (24-h collections) was analysed as received and after the addition of nedocromil sodium, at concentrations of 0.1 μg/ml and 10 μg/ml. The results of the analysis are shown in

Table 4
Results of the analysis of blank urine samples and of the same samples containing 0, 0.1 and 10 μg/ml added nedocromil sodium

Donor identification	Determined concentration (μg/ml)		
	0	0.1	10
1	0.000	0.093	10.06
2	0.000	0.093	9.99
3	0.000	0.098	10.14
4	0.003	0.096	10.10
5	0.000	0.092	9.98
6	0.001	0.094	10.09
7	0.002	0.098	10.15
8	0.005	0.097	9.62
Mean concentration (μg/ml)	0.001	0.095	10.02
Mean accuracy (%)	–	95	100
Standard deviation (μg/ml)	0.002	0.0024	0.172
Coefficient of variation (%)	–	2.5	1.7

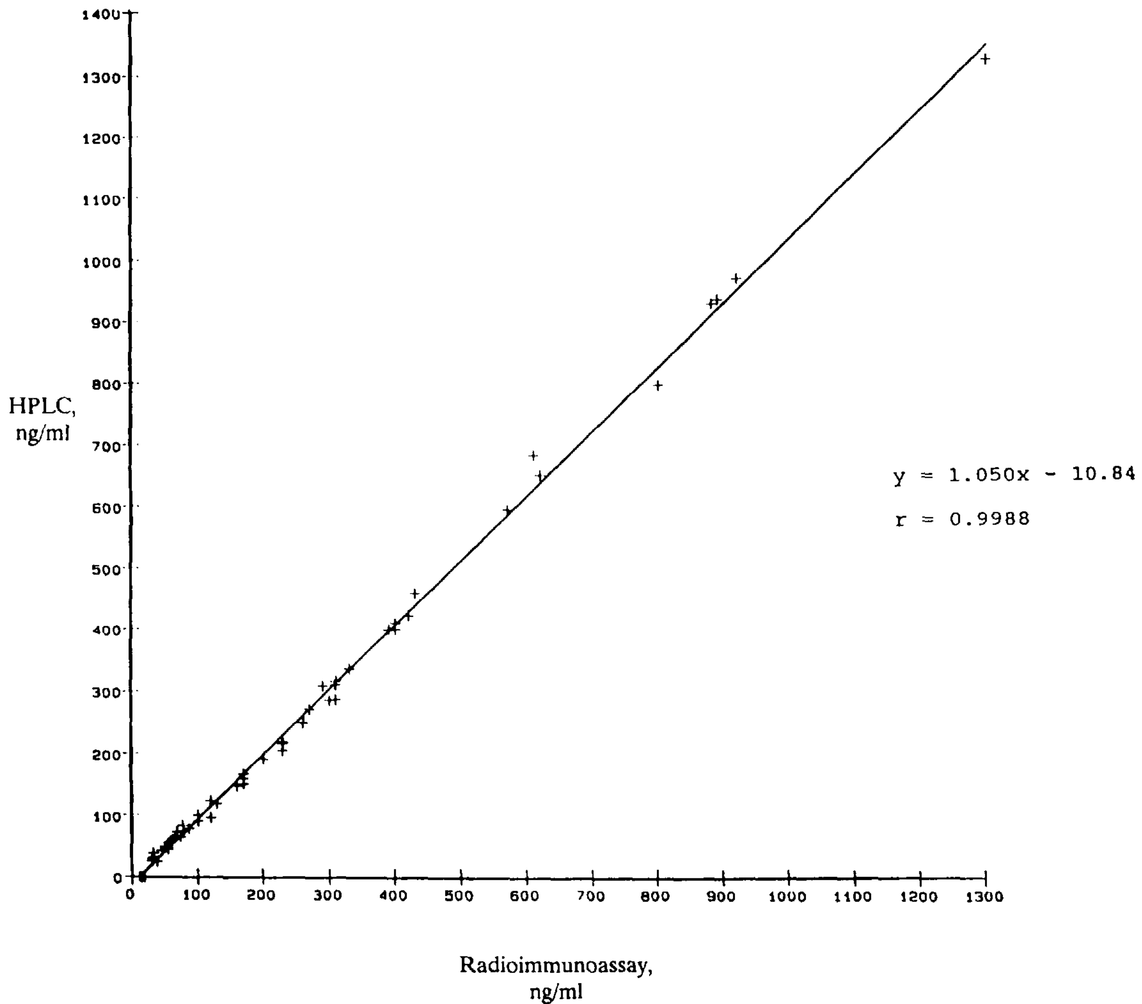

Fig. 5. Graph showing the correlation of results obtained using the column-switching method with those obtained by radioimmunoassay.

Table 4. The highest apparent concentration of nedocromil sodium determined in a blank urine sample was 0.005 μg/ml, and was probably due to detector noise. None of the individual urine samples appear to have imparted bias to the concentrations determined.

The specificity of the method, with respect to interference by drugs and various other compounds, was investigated by preparing solutions or suspensions (where solutions could not be made) of the substances, in mobile phase B, and injecting them directly onto the analytical column. The retention times of all the substances investigated, with respect to nedocromil sodium, are listed in Table 5. None of the drugs, or other compounds studied, produced peaks which interfered with the determination of nedocromil sodium.

The stability of nedocromil sodium in urine was investigated by the analysis of urine samples, containing added nedocromil sodium, after storage. Nedocromil sodium was stable for at least 22 days at ambient temperature and for at least 35 months at or below −20°C.

Urine samples, which had previously been analysed by radioimmunoassay [3], were ana-

Table 5
Retention times of drugs and other compounds relative to the retention time of nedocromil sodium

Compound	Relative retention time
Nedocromil sodium	1.00
Sodium cromoglycate	1.19
Acetylsalicylic acid	0.38
Salicylic acid	0.46
Terbutaline sulphate	0.34
Salbutamol sulphate	0.34
Isoprenaline sulphate	0.35
Theophylline	0.38
Caffeine	0.38
Saccharin sodium salt	0.54
Paracetamol	0.37
Beclomethasone dipropionate	0.34
Reproterol	0.36
Minocromil	0.56
Riboflavin	0.38
Quinoline yellow[a]	0.85, 1.14
Aspartame	0.37
Sorbitan trioleate	0.35
Menthol	0.35

[a] A single injection of quinoline yellow produced two peaks.

lysed using the column-switching procedure. The results of the two methods are compared graphically in Fig. 5.

4. Conclusions

An automated method for the determination of nedocromil sodium in human urine has been developed and the properties have been investigated. The method has acceptable accuracy and precision over the concentration range 0.02–10 μg/ml, and is specific in that no drugs or other compounds have been found which interfere with the determination of nedocromil sodium. The method has been successfully used to analyse samples from clinical studies.

Acknowledgement

The authors gratefully acknowledge the valuable technical assistance of A.I. Brutnell.

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