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Development and validation of a sensitive liquid chromatographic–tandem mass spectrometric method for the determination of cromolyn sodium in human plasma

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Abstract

Cromolyn sodium is a safe compound with potent anti-allergic properties when used locally or topically. Clinical data from systemic exposure is not available because of the poor GI absorption when given orally. In order to evaluate a new approach to enhance the absorption and bioavailability of cromolyn sodium, a sensitive assay was needed to support an oral-dose study in humans. This paper describes a liquid chromatographic–tandem mass spectrometric (LC–MS–MS) method for the analysis of cromolyn sodium in human plasma. The method consists of a two-step extraction with subsequent analysis using a high-performance liquid chromatography electrospray tandem mass spectrometer system. The compounds were eluted isocratically on a C_{18} column followed by a backflush. The total run time is 6 min. The standard curve of cromolyn sodium was over the range of 0.313 to 750 ng/mL with a lower limit of quantitation (LLOQ) of 0.313 ng/mL when 0.5 mL of plasma was used for analysis. The percent coefficient of variation (C.V.) for accuracy and precision (inter-assay and intra-assay) was less than 15% over the validated concentration range and the coefficients of determination, r^2, were >0.991577. The method is simple, sensitive, and selective, and has been successfully utilized for oral cromolyn sodium clinical studies.
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Keywords: Cromolyn sodium

1. Introduction

Cromolyn sodium is an anti-allergic drug effective for treating asthma and allergic rhinitis [1–3]. Clinical data from systemic exposure is not available because of the poor GI absorption when given orally [4,5]. The poor absorption of cromolyn sodium is attributed to its low lipophilicity, due possibly to its strongly acidic carboxyl groups [6]. Oral administration of cromolyn sodium may be able to achieve a prolonged therapeutic effect and improve compliance. Therefore, in order to reevaluate the absorption and bioavailability of cromolyn sodium, a sensitive assay was needed to support an oral-dose study in humans. Previously published bioassays and HLPC assays for cromolyn sodium lack specificity and sufficient sensitivity [7–10]. A quantitative LC–MS–MS method for the analysis of cromolyn so-

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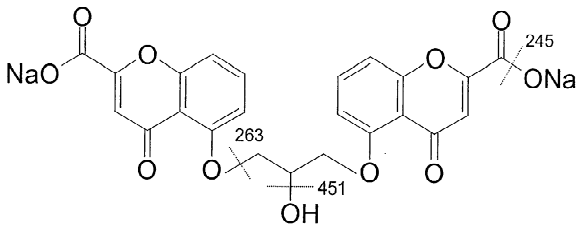
dium in human plasma using solid-phase extraction and an isotope-labeled internal standard has been reported [11]. This paper describes a simple, sensitive and specific LC–MS–MS method using liquid–liquid extraction and a non-isotope-labeled compound, warfarin, as internal standard for the determination of cromolyn sodium in human plasma.

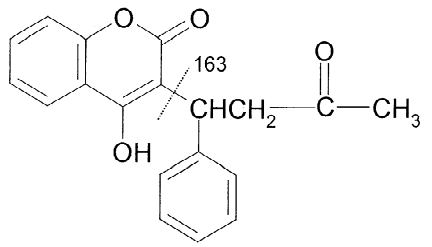
2. Experimental

2.1. Materials and reagents

Cromolyn sodium (purity 99.3%) was from Emisphere Technologies, Inc. (Tarrytown, NY, USA). Warfarin as the internal standard (I.S.) was obtained from USP (Rockville, MD, USA). The chemical structures of cromolyn sodium and the I.S. are shown in Fig. 1. Type I water was produced from an ultra-high-quality polishing system unit (UHQ-PS) (High Wycombe, Bucks, UK). Ethyl acetate, methyl *tert*.-butyl ether, methanol, and hydrochloric acid were from Fisher Scientific (St. Louis, MO, USA). Ammonium acetate was from Aldrich (Milwaukee, WI, USA), and trifluoroacetic acid (TFA) was from Burdick & Jackson (Muskegon, MI, USA). Blank human sodium citrate plasma was obtained from Bioreclamation Inc. (Hicksville, NY, USA) and was stored frozen at −20 °C. All mobile phase solvents were HPLC grade; all reagents were analytical reagent grade.



Cromolyn Sodium



Warfarin (IS)

Fig. 1. Chemical structures of cromolyn sodium and the internal standard (I.S.).

2.2. Calibration standards and quality control samples

Both cromolyn sodium and the I.S. were dried in vacuum over phosphorus pentoxide for 4 h before weighing. Standards and quality control samples (QCs) were made from two separate stock solutions (1 mg/mL in water, expressed as free base without correction for water and solvent content). Working calibration standards at concentrations of 0.313, 0.625, 2.5, 6.25, 62.5, 250, 500, and 750 ng/mL were prepared in blank plasma. Six levels of QC samples, 0.313, 0.625, 6.25, 62.5, 500, and 750 ng/mL, were prepared in plasma for the determination of inter-assay accuracy and precision. Working standards and QCs were prepared by appropriate dilution and the water-to-plasma ratio was kept relatively constant in each standard and QC sample. All standards and QCs were aliquoted and stored frozen at −20 °C with the clinical samples to be analyzed.

2.3. LC–MS–MS methods

LC–MS–MS analyses were performed using a Waters 2690 HPLC system (Milford, MA, USA) coupled to a Micromass Quattro LCZ triple-quadrupole mass spectrometer (Manchester, UK). The mass spectrometer was operated using an electrospray atmospheric pressure ionization source in positive ion mode (ESI^+) with multiple reaction monitoring (MRM). The analytical column was a MetaSil AQ C_{18}, 100×2 mm, 5 μm (Torrance, CA, USA). The mobile phase consisted of methanol–water (70:30) with 10 m*M* ammonium acetate and 0.02% TFA (pH 6.5) at an isocratic flow-rate of 0.2 mL/min. The sample injection volume was 20 μL

and the overall run time was 6 min per injection, including sample elution (3.5 min) and column backflushing (2.5 min). A Waters 590 programmable HPLC pump and an electrically controlled six-port HPLC valve (Catati, CA, USA) were used to backflush the column.

The sensitivity of MRM was optimized by infusing a mixture of 0.5 μg/mL cromolyn sodium and warfarin in the mobile phase. The capillary voltage was maintained at 3.75 kV. The cone and extractor voltages were set to 15 and 3 V, respectively. The desolvation and ion source temperatures were 350 and 60 °C, respectively. Ions were activated at a collision energy of 15 eV and at an indicated argon pressure of $2.7 \cdot 10^{-3}$ Torr. To assay the analytes, both quadrupoles were maintained at unit resolution and the transitions (precursor to daughter) monitored were m/z 469→245 for cromolyn sodium and m/z 309→163 for the I.S. The dwell time for each transition was 500 ms and the inter-channel delay was 30 ms.

MRM data were acquired and the chromatograms were integrated using MassLynx NT, version 3.2 software. Calibration curves were plotted as the peak area ratio of cromolyn sodium to the I.S. versus the corresponding nominal plasma concentration. A weighted $1/\text{concentration}^2$ quadratic regression was used to generate calibration curves from standards and calculate the sample concentrations.

2.4. Sample preparation

All samples, quality control samples, and standards with a sample volume of 0.5 mL were spiked with 100 μL of the I.S. (500 ng/mL in methanol), made slightly acidic by the addition of 1 mL of 0.1 *M* aqueous ammonium acetate (pH 6), and washed using 4 mL of ethyl acetate. The extraction tubes were shaken at high speed for 20 min followed by centrifugation at 4000 rpm for 20 min. The organic phase was discarded and 0.5 mL of 3 *M* hydrochloric acid in water was added to the tube, which was then vortexed for 3 min. Four milliliters of methyl *tert.*-butyl ether was added to the tube, shaken at high speed for 20 min, and centrifuged for 20 min at 4000 rpm. The organic phase was evaporated to dryness in a 45 °C water bath under a nitrogen stream. The samples were dissolved in 100 μL of mobile phase and vortexed for 1 min. After transfer into glass inserts of autosampler vials and centrifugation for 5 min at 4000 rpm, an aliquot of 20 μL of each sample was injected onto the LC–MS–MS system.

2.5. Validation of the LC–MS–MS method

The method was validated for accuracy, precision, sensitivity, specificity, calibration function, and reproducibility according to the FDA guidance for bioanalytical method validation for human studies [12] over a concentration range of 0.313 to 750 ng/mL using eight calibration standards and six replicates of QC samples at each concentration level in three separate batch runs. Each batch run also contained additional samples such as stability samples for processing and storage.

Analyte stability was tested using QC samples for multiple freeze–thaw cycles (F/T cycles), on the bench at room temperature (short-term stability), or frozen at −20 °C (long-term storage). Post-preparative stability and stock solution stability were also determined. The extraction recovery of cromolyn sodium was calculated by comparing the peak areas of extracted plasma standards to the peak areas of post-extraction plasma blanks spiked at corresponding concentrations. The overall absolute recovery from human plasma was determined by comparing the peak areas of extracted plasma standards to those prepared in mobile phase. The method specificity was evaluated by screening six lots of blank sodium citrate plasma.

3. Results and discussion

3.1. The LC–MS–MS method

Cromolyn sodium has carboxylic acid moieties. Therefore, electrospray (ESI) in negative ion mode was evaluated but was determined to be less sensitive than the positive ion mode. Atmospheric pressure chemical ionization (APCI) was also assessed but provided no advantages over ESI. The Q1 mass spectrum of cromolyn sodium showed protonated molecular ions $[M-2Na+3H]^+$ at m/z 469. The daughter scan spectrum of m/z 469 showed high-abundance fragment ions at m/z 245 (Fig. 2). The

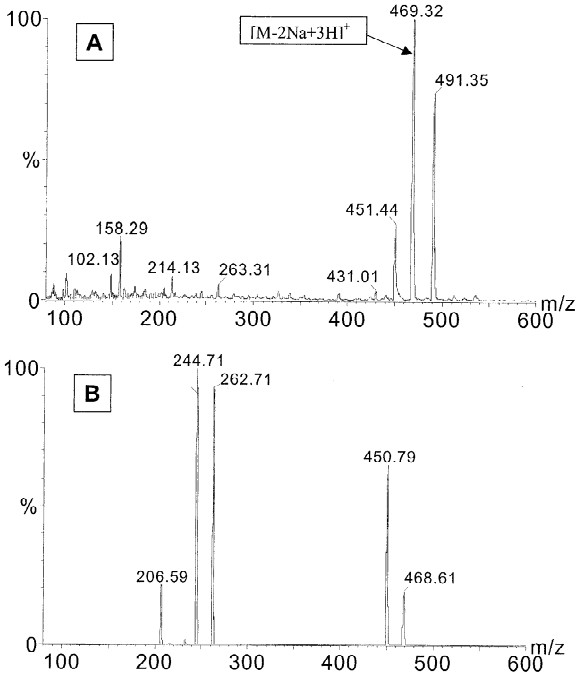


Fig. 2. (A) Q1 scan spectrum of cromolyn sodium. (B) Daughter scan spectrum of m/z 469 for cromolyn sodium.

fragmentation patterns are shown in Fig. 1. The ion transition of m/z 469→245 was chosen for multiple reaction monitoring of cromolyn sodium.

Warfarin, one of the compounds tested as the internal standard, demonstrated adequate recovery and offered good precision and accuracy for quantitation. Late-eluting peaks are one of the most vexing problems in LC separation [13] and are especially problematic in an LC–MS–MS method when the matrix peaks are not shown in the MRM channels. The late-eluting matrix can be eliminated by extending the run time or by flushing the column with a strong solvent. In order to shorten the run time in this method, the analytical column was backflushed with mobile phase at a higher flow-rate of 0.5 mL/min for 2.5 min to clean up the slowly eluting matrix after 3.5 min sample elution. A schematic of the column backflushing setup is shown in Fig. 3.

3.2. Specificity, sensitivity and calibration function

Human blank plasma samples from six different subjects were extracted and analyzed for cromolyn sodium as a true blank (double blank), or spiked with I.S., or with cromolyn sodium as a single blank. There were no endogenous peaks that interfered with the quantitation of cromolyn sodium or the I.S. There was no interference from the I.S. contributing to the cromolyn sodium m/z channels or from cromolyn sodium contributing to the I.S. m/z channel. The signal-to-noise ratio from an extracted lower limit of quantitation (LLOQ) sample (0.313 ng/mL) was at least 80. Another LC–MS–MS method used a relatively large amount of sample for analysis (1 mL) and achieved a LLOQ of 0.1 ng/mL [11]. There was no significant lot-to-lot variation in matrix effect and no carry-over from ULOQ to blank sample was

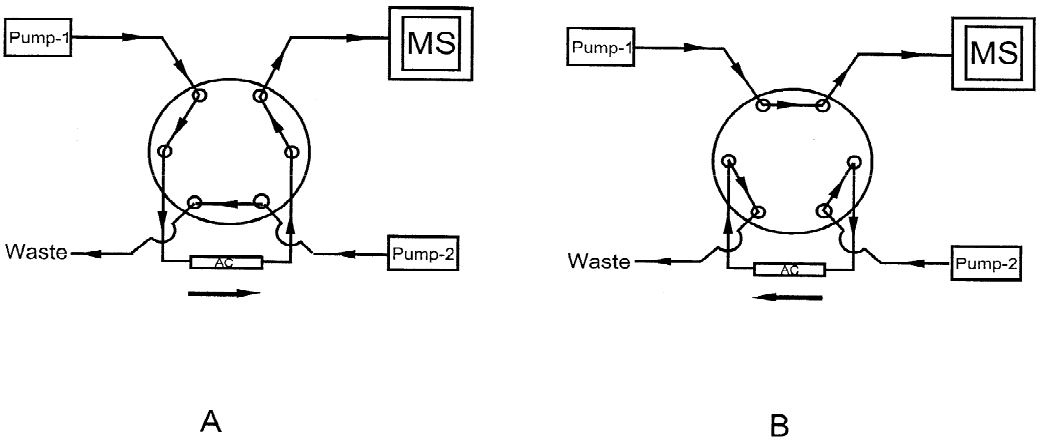


Fig. 3. Schematic of the column backflushing setup. (A) Sample elution. (B) Column backflushing. AC, analytical column.

observed. Various regression models were used to fit the calibration curve. A quadratic regression model ($y = ax^2 + bx + c$, where y is the peak area ratio of cromolyn sodium to I.S., x is the concentration of cromolyn sodium, and a, b and c are regression parameters) offered the best curve fitting. Calibration curves were over the concentration range of 0.313 to 750 ng/mL using the quadratic regression with a weighting factor of the reciprocal of the concentration squared ($1/x^2$). The regression parameters were between −1.40272e−6 and −1.50222e−6 for a, between 8.32131e−3 and 9.71557e−3 for b and between 2.52188e−4 and 3.99147e−4 for c. The coefficients of determination for cromolyn sodium were 0.991577 or better. Representative chromatograms of blank human plasma spiked with cromolyn sodium at 2.5 ng/mL and I.S., blank human plasma and blank human plasma spiked with I.S. only are shown in Figs. 4–6.

3.3. Precision, accuracy and dilution integrity

Table 1 shows the validation data on the accuracy and precision of each standard concentration. The inter-assay coefficient of variation (C.V.) for the back-calculated calibration standards ranged from 3.4 to 10.1% and the nominal concentrations ranged from 90.2.0 to 106.4%. The precision and accuracy data for QCs are summarized in Table 2. Inter-assay C.V. values were less than 13.4% and the nominal concentrations ranged from 90.5 to 109.1%. Intra-assay C.V. values were less than 14.8% and the nominal concentrations ranged from 88.2 to 109.4%. These C.V. and nominal concentration values indicated reproducible LC–MS–MS conditions and that

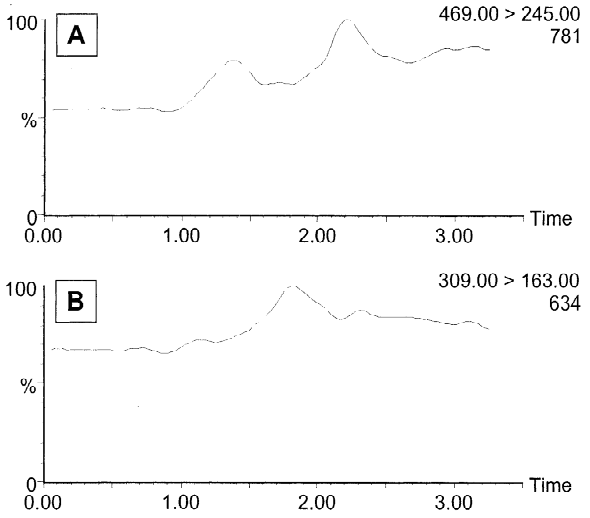


Fig. 5. Chromatograms of an extracted blank human plasma sample. (A) Cromolyn sodium channel: m/z 469→245. (B) I.S. channel: m/z 309→163.

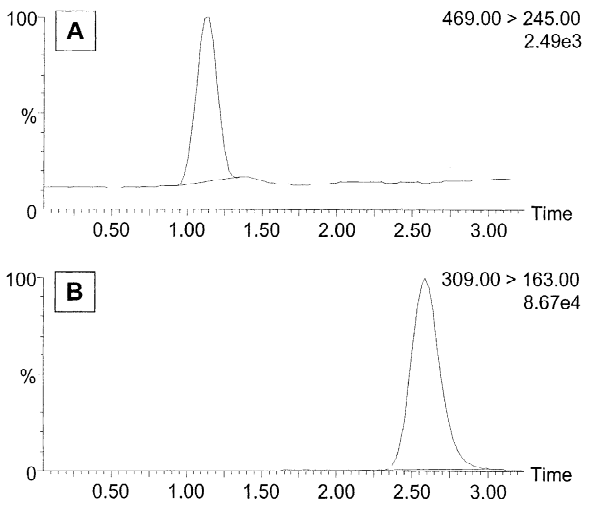


Fig. 4. Chromatograms of an extracted human plasma spiked with cromolyn sodium at 2.5 ng/mL. (A) Cromolyn sodium channel: m/z 469→245. (B) I.S. channel: m/z 309→163.

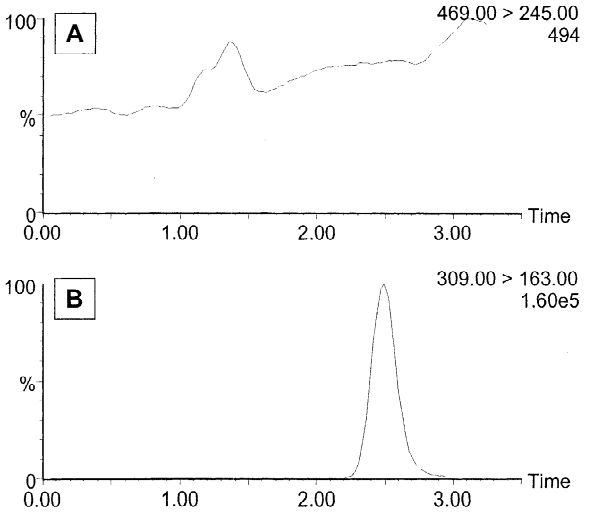


Fig. 6. Chromatograms of an extracted human plasma spiked with internal standard only. (A) Cromolyn sodium channel: m/z 469→245. (B) I.S. channel: m/z 309→163.

Table 1
Precision and accuracy of calibration standards ($n=3$)

Nominal (ng/mL)	Mean	SD	C.V. (%)	% Nominal
0.313	0.293	0.010	3.4	93.6
0.625	0.564	0.024	4.3	90.2
2.5	2.46	0.02	8.1	98.4
6.25	5.75	0.58	10.1	92.0
62.5	63.4	3.2	5.0	101.5
250	259	11	4.2	103.6
500	461	42	9.1	92.1
750	798	50	6.3	106.4

the assay is consistent and reliable. For partial volume analysis, QC samples (750 ng/mL) were diluted 10-fold with blank plasma prior to extraction. The dilution integrity data showed a C.V. of 9.5% with a nominal concentration of 96.4%. These results support sample dilution up to 10-fold for analysis.

3.4. Recovery and matrix effect

The extraction recovery of cromolyn sodium was determined by comparing the peak areas of extracted plasma standards at 0.625, 6.25 and 62.5 ng/mL to the peak areas of post-extraction plasma blanks spiked at corresponding concentrations. Extraction recovery of cromolyn sodium from human plasma ranged from 42.6 to 51.4% with a C.V. (%) of less than 10.9%, and the overall absolute recovery ranged from 41.8 to 55.2% with a C.V. (%) of less than 9.6%, indicating a relatively low but consistent recovery with no matrix suppression of cromolyn sodium. The overall recovery of the I.S. spiked into plasma standards at 500 ng/mL was 64.8% with a C.V. (%) of 7.4%. In comparison with some solid-phase extraction methods which offer better recoveries for cromolyn sodium [11], the relatively low recoveries of both cromolyn sodium and the I.S. were probably due to losses during the two-step extraction for sample clean-up and their low lipophilicity.

Table 2
Precision and accuracy of quality control samples

Nominal (ng/mL)	Mean	SD	C.V. (%)	% Nominal
Intra-assay ($n=5$)				
0.313	0.322	0.046	14.3	102.9
0.625	0.674	0.100	14.8	107.8
6.25	6.84	0.51	7.5	109.4
62.5	63.8	6.6	10.3	102.1
500	441	33	7.5	88.2
750	697	31	4.4	92.9
Inter-assay ($n=3$)				
0.313	0.287	0.018	6.3	91.7
0.625	0.604	0.081	13.4	96.6
6.25	6.82	0.57	8.4	109.1
62.5	65.4	7.1	10.9	104.6
500	466	54	11.6	93.2
750	679	87	12.8	90.5

3.5. Stability of the analytes

Stability testing of the analytes was designed to cover anticipated conditions for clinical samples. Stability data are summarized in Table 3. Briefly, three freeze–thaw cycles and ambient temperature storage of the QC samples up to 4 h prior to sample preparation appeared to have no effect on the quantitation of cromolyn sodium. QCs stored in a freezer at −20 °C remained stable for at least 1 month. Extracted analytes were allowed to stand at ambient temperature in mobile phase for 24 h prior to LC–MS–MS analysis, with no observed effect on quantitation. Stability of stock solutions was also investigated. Stock solutions of cromolyn sodium in water at a nominal temperature of 4 °C were stable for 1 month.

3.6. Applicability of method

The method was successfully utilized for determining the plasma concentration of cromolyn sodium in a single dose, open-label, escalating oral dose clinical study. The mean cromolyn sodium plasma profile from eight healthy subjects is shown in Fig. 7.

4. Conclusions

The LC–MS–MS method for the determination of cromolyn sodium in human plasma showed good inter-assay and intra-assay precision and accuracy over the calibration range of 0.313 to 750 ng/mL. The method is simple, sensitive and selective, and

Table 3
Stability of cromolyn

	Concentration (ng/mL)					
	0.625	6.25	62.5	250	500	750
4 h at 25 °C						
Mean ($n=3$)	0.546	6.03	NE	236	NE	NE
% C.V.	2.4	2.8	NE	2.9	NE	NE
% Nominal	87.4	96.5	NE	94.4	NE	NE
1 month at −20 °C						
Mean ($n=3$)	0.707	6.84	75.5	NE	NE	769
% C.V.	18.0	8.2	4.7	NE	NE	5.6
% Nominal	113.1	109.4	120.7	NE	NE	102.6
Three freeze–thaw cycles						
Mean ($n=3$)	0.548	6.43	NE	236	NE	NE
% C.V.	6.9	6.6	NE	4.7	NE	NE
% Nominal	87.7	99.0	NE	90.7	NE	NE
24 h post-preparative						
Mean ($n=3$)	0.656	5.85	61.0	NE	480	NE
% C.V.	4.5	4.0	9.5	NE	6.5	NE
% Nominal	105.0	93.6	97.6	NE	95.9	NE

NE, not evaluated.

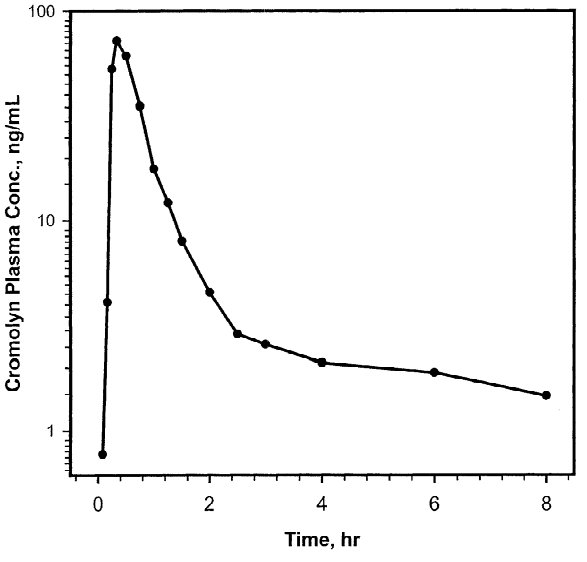


Fig. 7. Mean plasma profile of cromolyn sodium from eight healthy subjects after receiving an oral dose of 150 mg of cromolyn sodium with 200 mg of a delivery agent.

has been successfully utilized for oral cromolyn sodium clinical studies.

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