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DETERMINATION OF AZELASTINE AND DESMETHYLAZELASTINE IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A manual-injection liquid chromatographic method using fluorescence detection permitted determination of a new antiasthmatic drug, azelastine, and its desmethyl metabolite extracted from human plasma. Reliable quantitation was achieved to at least 0.3 ng/ml for each analyte. No interference was seen in co-chromatography of sixteen other substances, which were potential co-medications (or their metabolites) as used in standard asthma or allergy treatment.

INTRODUCTION

Azelastine is a pharmacologically unique compound of novel structure (Fig. 1) with a broad spectrum of antiallergic and antiasthmatic activities following oral or aerosol administration. The results of preclinical and clinical studies have recently been reviewed [1].

A gas chromatographic–mass spectrometric (GC–MS) assay for the drug in dog plasma in the concentration range 10–500 ng/ml was previously published [2], however, for more routine work in clinical drug development an alternative assay was needed. With the assumed effective dose varying between 0.5 and 8 mg the human plasma levels were expected to be in the low ng/ml range. Maximum plasma drug concentrations of approximately 2 ng/ml and less were indicated by radioimmunoassay after a 4-mg oral dose [3]. In biotransformation studies in animals the presence of multiple metabolites was observed [4,5].

In this communication we describe a reversed-phase high-performance liquid chromatographic (HPLC) assay for human plasma based on fluorescence detection, which allows quantitation of azelastine and the desmethyl metabolite (Fig.

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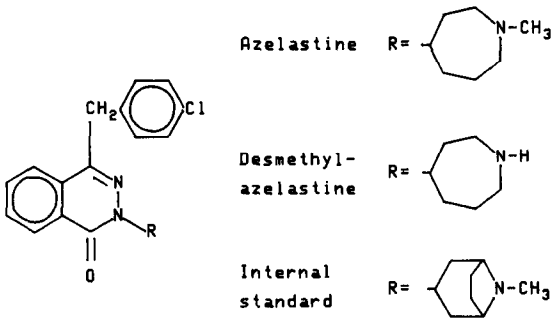

Fig. 1. Structures of azelastine, desmethylazelastine and internal standard.

1) in the range 0.3–3.33 ng/ml. This assay has been applied to human Phase I and pharmacokinetic studies.

EXPERIMENTAL

Chemicals

Azelastine hydrochloride, 4-(*p*-chlorobenzyl)-2-(hexahydro-1-methyl-1H-azepinyl-4-yl-1(2H)-phthalazinone monohydrochloride, [^{14}C]azelastine hydrochloride (labeled in the 1,4-positions of the phthalazinone ring [6]), desmethylazelastine hydrobromide, 4-(*p*-chlorobenzyl)-2-[perhydroazepinyl-(4)]-1(2H)-phthalazinone hydrobromide, and the internal standard, 4-(*p*-chlorobenzyl)-2-[N-methyl-2,6-ethanopiperidinyl-(4)]-1-(2H)-phthalazinone hydrochloride hydrate, were obtained from Degussa Pharma Gruppe (Frankfurt, F.R.G.). Glass-distilled solvents (acetonitrile, chloroform, *n*-hexane, isopropanol, methanol, tetrahydrofuran), all Burdick & Jackson, were purchased through American Scientific Products (McGaw Park, IL, U.S.A.). Analytical-grade sodium hydroxide, hydrochloric acid (Mallinckrodt, Paris, KT, U.S.A.), acetic acid (J.T. Baker, Phillipsburg, NJ, U.S.A.), triethylamine (Pierce, Rockford, IL, U.S.A.) were used in this study. Heparinized human plasma was purchased from Biological Specialty Corp. (Lansdale, PA, U.S.A.).

The following compounds were listed in the drug interference study and were obtained from the sources indicated: 6-α-methylprednisolone, hydrocortisone, 1,3-dimethyluric acid, 1,7-dimethylxanthine, 1-methylxanthine, acetaminophen, 1-methyluric acid, 3-methylxanthine, caffeine, racemic pseudoephedrine hydrochloride, acetylsalicylic acid (Sigma, St. Louis, MO, U.S.A.); isoproterenol hydrochloride (Aldrich, Milwaukee, WI, U.S.A.); albuterol sulfate (Sandoz, East Hanover, NJ, U.S.A.); terbutaline sulfate (Ciba-Geigy Pharmaceuticals, Summit, NJ, U.S.A.); theophylline (Pfaltz & Bauer, Stamford, CT, U.S.A.); and cromolyn sodium (Fisons, Bedford, MA, U.S.A.).

Instrumentation

Experiments were conducted to determine optimal conditions for fluorescence detection and recovery of drug. A McPherson Model FL750 spectrofluorometer

with scan controller, Model 789B (McPherson, Acton, MA, U.S.A.), interfaced to an HP 3357 laboratory automation system (Hewlett-Packard, Avondale, PA, U.S.A.) were used for studying fluorescence characteristics of analytes and internal standard. The extraction parameters were monitored using [^{14}C]azelastine hydrochloride and a Model LS 9000 liquid scintillation counter from Beckman Instruments (Fullerton, CA, U.S.A.). The liquid chromatographic system included a Waters Assoc. Model 6000A pump and a Model 720 systems controller (Milford, MA, U.S.A.) with a 100-μl Rheodyne fixed-loop injector (Berkeley, CA, U.S.A.). A Schoeffel Model 970 variable-wavelength fluorescence monitor (Spectros, Ramsey, NJ, U.S.A.) in conjunction with the HP 3357 laboratory automation system was used for compound detection and data analysis.

Optimization of fluorescence detection

A series of tests were done to find the optimum conditions for fluorescence monitoring by subjecting each compound to emission scans over the region 215–615 nm at fixed excitation wavelengths of 200, 215, 250, 300 and 350 nm, respectively. Each compound was dissolved in mobile phase to achieve a concentration of 50 ng/ml. Spectra were obtained using a McPherson fluorometer with a deuterium source and a 6-μl quartz flow cell. Operating conditions were ambient temperature with the sensitivity set at 0.03, photomultiplier high voltage (PMT/HV) set at 680 V, slit bandwidth of 8 nm and a scanning speed of 200 nm/min. Data were collected and processed by the HP 3357 laboratory automation system.

Optimization of drug extraction from human plasma

[^{14}C]Azelastine hydrochloride stock solutions were prepared in distilled water and in human plasma at 10 ng/ml. A 1.0-ml aliquot of each stock solution in 10 ml Aquasol was counted for 10 min along with the scintillation solution blank as reference. Single and multiple extractions were then carried out on duplicate 3-ml plasma aliquots of stock solution using various solvents or solvent mixtures. Each plasma was made alkaline with 1 ml of 1.0 *M* sodium hydroxide, and 15 ml of extracting solvent were added. Each sample was vortexed for 1 min and centrifuged for 10 min at about 1000 *g* (10°C). The above steps were repeated for the double extractions and the entire organic phases were evaporated under nitrogen gas. After evaporation the residues were reconstituted in 10 ml Aquasol for liquid scintillation counting or in 100 μl mobile phase for HPLC determination.

Investigation of potential interfering substances

The compounds listed in Table V were prepared in and diluted with 0.012 *M* hydrochloric acid (solvent 1) or mobile phase (solvent 2) to the stated concentrations given. Analysis was performed as described under *Chromatographic conditions.*

Preparation of solutions and standards

Stock solutions of the internal standard were prepared in triplicate each containing 10 mg per 100 ml in 0.012 *M* hydrochloric acid. These were diluted with

0.012 *M* hydrochloric acid to give working solutions containing 100 ng/ml. Mixture stock solutions of 10 mg azelastine hydrochloride and 10 mg desmethylazelastine hydrobromide were prepared in triplicate using 100 ml of working internal standard (I.S.) solution. Final working mixture standards contained 0 (blank), 5, 10, 25, 50 and 100 ng/ml of each analyte and 100 ng/ml I.S. Using 100-μl aliquots of the mixture working standards to fortify 3 ml plasma, corresponding triplicate plasma standards containing 0, 0.17, 0.33, 0.83, 1.67 and 3.33 ng/ml of each analyte and 3.33 ng/ml I.S. were obtained for use in various calibration curves.

Extraction procedure

To 3 ml plasma in a 50-ml polypropylene centrifuge tube (Miles Labs., Naperville, IL, U.S.A.) 150 μl of 1% (v/v) acetic acid were added with brief manual mixing. The sample was made alkaline (pH 12) with 1 ml of 1.0 *M* sodium hydroxide and extracted with two successive 15-ml portions of *n*-hexane–isopropanol (97:3, v/v). The pooled organic phases were centrifuged for 6 min at approximately 1000 *g* to remove any separated solids or traces of aqueous phase. The analytes in the transferred organic layer were then back-extracted with 120 μl of 0.012 *M* hydrochloric acid under vigorous vortex-mixing for 2 min. Following phase separation at room temperature for about 10 min, a 100-μl portion of the lower acid aqueous phase was removed and manually injected for HPLC analysis.

Chromatographic conditions

A 250 mm $\times$ 4.6 mm I.D. Altex Ultrasphere 5-μm ODS reversed-phase column (Rainin Instrument, Emeryville, CA, U.S.A.) was operated at 30°C (Schoeffel column oven Model LC250/3) at a flow-rate of about 0.8 ml/min corresponding to approximately 136 bar pressure. The mobile phase consisted of tetrahydrofuran–water (30:70, v/v) with 0.3% (v/v) triethylamine (relative pH 2.3–2.4 with orthophosphoric acid as determined using a glass electrode), which was unfiltered but sonified prior to use. Eluent was monitored with detector settings of 213 nm excitation wavelength, emission cut-off filter of 360 nm and detector sensitivity at 580 V, 200 a.c. at 1020.

RESULTS AND DISCUSSION

Optimization of fluorescence detection

From previous work the ultraviolet absorbance maximum of azelastine and related compounds had been established at about 213 nm and it was also determined that these compounds did exhibit fluorescence when excited at this wavelength. As shown in Fig. 2 the optimum wavelength of excitation was 215 nm and it produced maximum fluorescence emission at 360 nm for all these compounds. Use of a 360-nm cut-off filter enhanced detector selectivity and improved the signal-to-noise ratio.

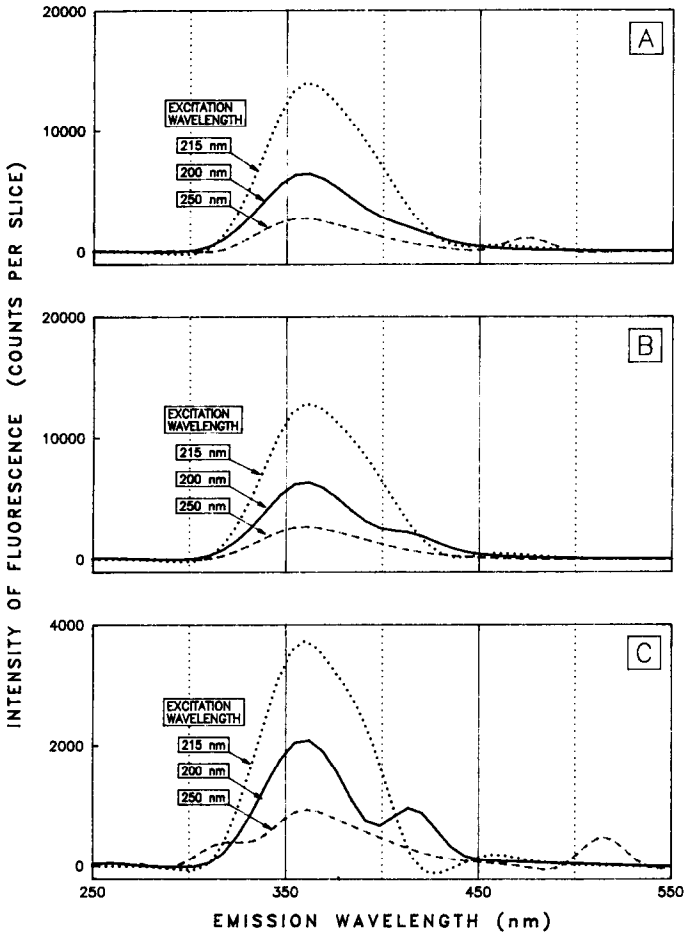

Fig. 2. Effect of excitation wavelength on the fluorescence intensity of the emission at various wavelengths for azelastine hydrochloride (A), desmethylazelastine hydrobromide (B) and the internal standard (C).

Optimization of drug extraction from human plasma

The procedure peviously reported [2] for extracting azelastine required modifications to make it suitable for a liquid versus gas chromatographic system and fluorescence rather than mass spectral detection. As shown in Table I, a series of extraction experiments were conducted with ^{14}C-labeled drug. Single extractions with chloroform produced low drug recovery and extracted a considerable number of unwanted endogenous compounds. Hexane extractions proved cleaner via chromatographic monitoring but drug recovery was still poor. The addition of increasing amounts of isopropanol to hexane gave improved recovery of azelastine from plasma. A second extraction with 3 or 5% isopropanol in hexane gave about 10% additional drug recovery for a combined total recovery approaching 94%. The overall cleaner chromatographic profile observed with the lower amount of alcohol (i.e. 3%) in the extracting solvent, provided the best compromise between good drug recovery and acceptable chromatography.

TABLE I

OPTIMIZATION OF PLASMA EXTRACTION PROCEDURE USING [^{14}C]AZELASTINE HYDROCHLORIDE

Experimental conditions		dpm	Recovery (%)
Reference solutions			
Aquasol blank		0	—
[^{14}C]Azelastine hydrochloride in water		1173*	100.0
[^{14}C]Azelastine hydrochloride in plasma		1179*	100.0
Single extractions of [^{14}C] azelastine hydrochloride in plasma			
Chloroform		350	29.7
Hexane		667	56.6
Hexane–isopropanol (99:1)		812	68.9
Hexane–isopropanol (98:2)		876	74.3
Hexane–isopropanol (97:3)		924	78.4
Hexane–isopropanol (96:4)		928	78.7
Hexane–isopropanol (95:5)		972	82.4
Multiple extractions of [^{14}C] azelastine hydrochloride in plasma			
Hexane–isopropanol (97:3)	Extraction 1	984	83.5
	Extraction 2	122	10.4
Hexane–isopropanol (95:5)	Extraction 1	936	79.4
	Extraction 2	108	9.2

*dpm·3.

Studies on the chromatographic separation of compounds

Very early chromatographic work with azelastine employed a 10-μm particle size bonded silica material eluted with acetonitrile–methanol–water (37:10:53). Following the isolation and identification of the desmethyl metabolite, it was shown that the above conditions would not resolve parent drug and metabolite. As summarized in Table II, a new mobile phase system was developed using mixtures of tetrahydrofuran–water with triethylamine (TEA) on a 5-μm particle size bonded silica packing. The extrema of conditions are listed, however, stepwise adjustments of each were made to maximize the separation of analytes and the internal standard in the new mobile phase.

Method performance parameters

The separation accomplished with the conditions listed in Table II is shown in Fig. 3. Linearity of detector response was achieved over the stated concentration range as evidenced by the correlation coefficients $r^2 > 0.99$ for all curves. The absolute recovery of azelastine, internal standard and desmethylazelastine for this method was found to be 65, 55 and 60%, respectively. This was determined by comparing peak heights obtained from fortified plasma samples with those from directly injected standard solutions of identical concentrations. The apparent discrepancy in recovery of azelastine extracted from plasma reported above as 65% versus that obtained in the radiolabeling study (Table I) can be accounted for. First, the radiolabeling work measured only the combined hexane–isopropanol extracts. It did not include the back-extraction step with 120 μl

TABLE II

OPTIMIZATION OF HPLC SEPARATION

Parameter	Initial conditions	Final conditions
Mobile phase	Tetrahydrofuran–water (48:52) with 1 ml TEA	Tetrahydrofuran–water (30:70) with 3 ml TEA
pH with orthophosphoric acid	2.6–2.7	2.3–2.4
Flow-rate (ml/min)	0.7	0.8
Temperature (°C)	35	30
Detector time constant (s)	3	4
Retention time (min)		
Azelastine	9.3	9.4
Internal standard	9.9	10.2
Desmethylazelastine	10.6	11.2

of dilute acid, which was a clean-up procedure employed later to improve HPLC. In fact, the approximate final volume of aqueous acid phase actually increased to about 150 μl with partitioning of some isopropanol into it. Finally, only 100 μl of the total 150-μl phase was used and, therefore, the uncorrected analytical recovery appeared to be lower.

The intra-day precision (coefficient of variation, C.V.) for determination of the drug (Table III) and metabolite (Table IV) in fortified standard plasma samples varied from 1.8 to 3.9% and 2.9 to 7.7%, respectively. The accuracy (relative mean error) varied from 0.7 to 3.8% and 0.5 to 9.5%, respectively.

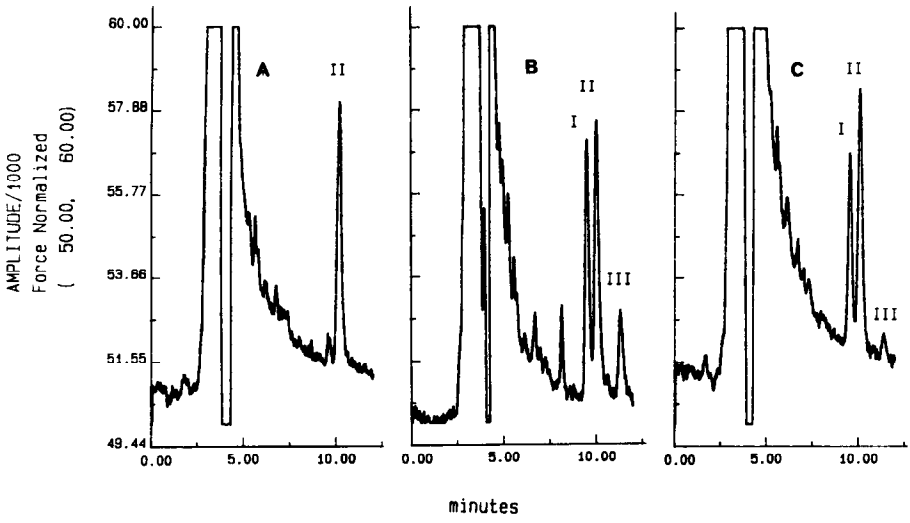

Fig. 3. Chromatograms of (A) a blank plasma sample with I.S. (II); (B) a blank plasma sample fortified with 0.83 ng/ml azelastine hydrochloride (I), 0.83 ng/ml desmethylazelastine hydrobromide (III) and 3.33 ng/ml internal standard (II); and (C) a human plasma sample obtained from a subject 1.0 h after a 4.4-mg dose of azelastine hydrochloride.

TABLE III

PRECISION AND ACCURACY DATA FOR THE DETERMINATION OF AZELASTINE IN HUMAN PLASMA

Stated concentration* (ng/ml)	Determined concentration* (ng/ml)	Coefficient of variation (%)	Relative mean error (%)
Intra-day (n=3)			
0.33	0.34	3.9	3.1
0.83	0.86	2.9	3.0
1.67	1.61	2.6	3.8
3.33	3.35	1.8	0.7
Inter-day (n=4)			
0.17	0.17	6.6	0.1
0.33	0.34	10.9	2.5
0.83	0.82	9.7	1.7
1.67	1.67	10.2	0.3

*Expressed as hydrochloride salt.

Similarly, the inter-day C.V.s for drug (Table III) and metabolite (Table IV) were 6.6–10.9% and 4.8–11.5%, respectively. The corresponding accuracy values were 0.1–2.5% and 0.1–15.7%, respectively. We consider these values quite acceptable for measurements of concentrations in the sub-nanogram range (0.79–7.9 pmol).

The limit of detection for this method was arbitrarily set at 0.33 ng/ml (the lowest concentration of the intra-day standard curve). However, lower concentrations (to 0.1 ng/ml) could be quantitated but with a loss of precision and accuracy.

The stability of human plasma samples during freezer storage was determined

TABLE IV

PRECISION AND ACCURACY DATA FOR THE DETERMINATION OF DESMETHYLAZELASTINE IN HUMAN PLASMA

Stated concentration* (ng/ml)	Determined concentration* (ng/ml)	Coefficient of variation (%)	Relative mean error (%)
Intra-day (n=3)			
0.33	0.35	2.9	9.5
0.88	0.96	4.7	9.5
1.67	1.67	7.7	4.3
3.33	3.30	7.3	0.5
Inter-day (n=4)			
0.17	0.19	11.5	15.7
0.33	0.33	4.8	0.1
0.83	0.78	6.4	7.3
1.67	1.69	7.3	1.5

*Expressed as hydrobromide salt.

TABLE V

LIST OF POTENTIAL CHROMATOGRAPHIC INTERFERENCES

Values in parentheses are the solvents used: 1 = 0.012 *M* hydrochloric acid; 2 = mobile phase.

Compound	Retention time (min)	Injection (μg per 100 μl solvent)	Relative peak height
6-α-Methylprednisolone	2.64	2 (2)	0.30
Hydrocortisone	2.65	0.5 (2)	0.30
1,3-Dimethyluric acid	2.70	20 (2)	> 1.00
1,7-Dimethylxanthine	2.72	20 (2)	> 1.00
1-Methylxanthine	2.76	20 (2)	> 1.00
Acetaminophen	3.00	20 (1)	> 1.00
dl-Isoproterenol hydrochloride	3.00	0.5 (1)	> 1.00
1-Methyluric acid	3.00	20 (2)	> 1.00
3-Methylxanthine	3.00	20 (2)	> 1.00
Albuterol sulfate	3.18	0.02 (1)	> 1.00
Terbutaline sulfate	3.52	0.02 (1)	> 1.00
Theophylline	3.53	20 (1)	> 1.00
Caffeine	3.54	20 (1)	> 1.00
Cromolyn sodium	3.65	0.1 (1)	0.35
Pseudoephedrine hydrochloride	4.29	0.5 (1)	> 1.00
Acetylsalicylic acid	7.60	2 (1)	> 1.00
Azelastine hydrochloride	9.57	0.01 (1)	1.00
Internal standard	10.25	0.01 (1)	0.25
Desmethylazelastine hydrobromide	10.99	0.01 (1)	0.75

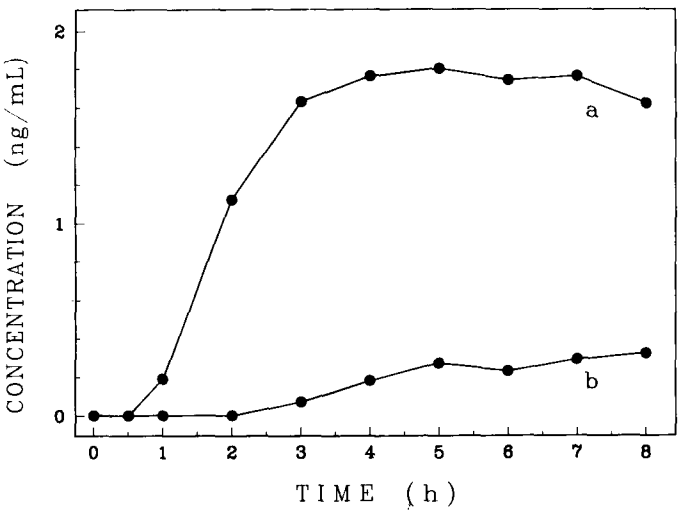

Fig. 4. Changes in plasma concentrations of azelastine (a) and desmethylazelastine (b) with time after an oral dose of 4.4 mg azelastine hydrochloride.

up to 88 days. An analysis of variance of these results showed that no statistically significant change had occurred. The results of a co-medication interference study (Table V) indicated no interference by any of these major drugs and some of their metabolites because none of these substances eluted in the drug region.

Fig. 3 represents typical chromatograms of human plasma samples. The one obtained from a subject 1 h after a 4.4-mg oral dose of azelastine hydrochloride (C) was found to contain 0.59 ng/ml of azelastine and 0.16 ng/ml of desmethylazelastine, both calculated as free base. In Fig. 4 the mean plasma drug and metabolite concentration versus time curve is shown for a group of four patients dosed with 4.4 mg of drug. Over 1000 plasma samples from clinical studies have been analyzed with this assay. Approximately twenty samples could be processed and analyzed in a typical 8-h work-day by two analysts because the assay requires very careful and accurate sample manipulation.

For assays of samples from patients dosed with less than 2 mg of drug and for highly accurate pharmacokinetic studies, a new microbore HPLC assay is under development.

REFERENCES

1 J.P. Kemp, E.O. Meltzer, H.A. Orgel, M.J. Welch, G.A. Bucholtz, E. Middleton, S.L. Spector, J.J. Newton and J.L. Perhach, J. Allergy Clin. Immunol., in press.
2 J. Hasegawa, Y. Tomono, M. Tanaka, T. Fujita, K. Sugiyama and N. Hirose, Arzneim.-Forsch., 31 (1981) 1215.
3 G. Peter, Degussa Pharma, Frankfurt, personal communication.
4 K. Tatsumi, T. Ou, H. Yamada and H. Yoshimura, Jpn. J. Pharmacol., 30 (1980) 37.
5 K. Tatsumi, H. Yamada, H. Yoshimura, Y. Nishizawa, M. Sakai, H. Mizuo and C. Yamato, Hiroshima J. Med. Sci., 33 (1984) 669.
6 G. Scheffler, H. Beckel and E. Schaumloffel, Arzneim.-Forsch., 33 (1983) 1515.