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Determination of alosetron in human plasma or serum by high-performance liquid chromatography with robotic sample preparation

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Abstract

A method of analysis for the determination of alosetron in human plasma or serum has been developed. The method was fully automated using a laboratory robot in order to improve analytical precision, efficiency and safety. The assay involved solid-phase extraction with reversed-phase HPLC separation and fluorescence detection. A validation exercise over the concentration range of 0.1 to 20 ng/ml demonstrated the selectivity, linearity, sensitivity, accuracy, precision, extraction efficiency, ruggedness and stability of the method. The method has been applied in support of numerous human pharmacokinetic/biopharmaceutic studies over the last five years.

Keywords: Alosetron; Robotic sample preparation

1. Introduction

During the last several years evidence has been found for a role of 5-HT_3 (serotonin type 3) receptors in the regulation of dopaminergic neurotransmission. The 5-HT_3 antagonists have been shown to antagonize the consequences of raised mesolimbic dopamine activity, such as from injection of amphetamine or dopamine into the nucleus accumbens. The effects of the 5-HT_3 antagonists are only evident when the activity of the mesolimbic dopamine system is raised; there is no observable effect on normal locomotor activity [1].

Alosetron (Fig. 1), a potent and selective 5-HT_3 receptor antagonist, has been shown to antagonize the consequences of raised mesolimbic dopamine activity [2]. Based on results of this and other models for antipsychotics, alosetron was targeted for investigation in the treatment of schizophrenia. A sensitive and selective method for its determination in plasma or serum was developed in order to support the clinical development of alosetron, also known as GR68755. The method was developed originally using robotic sample analysis, in order to process large numbers of samples more precisely, efficiently and safely.

This paper reports an HPLC method for the determination of alosetron in plasma or serum which has been performed both as a fully automated process or manually, by different analysts and in different laboratories, over the last five years. The

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method was validated as a selective, sensitive, accurate, precise and rugged assay.

2. Experimental

2.1. Reagents

Alosetron hydrochloride, the internal standard GR87442 (Fig. 1), and available metabolites were supplied by the Central Analytical Services Department of Glaxo Group Research. A.R. grade ammonium acetate and glacial acetic acid were acquired from Mallinckrodt (Paris, KY, USA). High purity methanol, isopropanol, acetonitrile and tetrahydrofuran were obtained from Burdick and Jackson (Muskegon, MI, USA). LRC Bond Elut 100-mg ethyl (C_2) extraction columns were purchased from Varian (Harbor City, CA, USA).

2.2. Sample preparation

The robot isolated alosetron and the internal standard from plasma and serum samples by solid-phase extraction using 100-mg C_2 extraction columns. After a column was loaded into the solid-phase extraction station, the column sorbent bed was activated with 1 ml of isopropanol and rinsed with 1 ml of buffer (0.01 *M* ammonium acetate adjusted to pH 4.0 with glacial acetic acid). A series of stepper-motor driven syringes delivered solvents onto the column bed through teflon tubing connected to the station cap. The cap pneumatically closed over the top of the column forming an air-tight seal. Another tubing line from the cap was connected to a regulated nitrogen source to force the solvents through the column with variable positive pressure.

While the column was being conditioned (41 kPa of positive pressure applied to the extraction column), the robot pipetted 1.1 ml of sample from its original container stored in a refrigerated rack. The aliquot was dispensed into a fresh, tared test tube in the balance and the weight was recorded by printer output. The robot then added 1 ml of buffer containing internal standard (10 ng/ml GR87442). It vortexed the sample mixture and pipetted 2 ml onto the conditioned extraction column. After loading the sample (55 kPa), the extraction column was washed with 2 ml of buffer (83 kPa), dried with nitrogen for 30 s, and washed with 2 ml of acetonitrile (28 kPa). Alosetron and GR87442 were eluted into a clean collection tube using two 2 ml aliquots of acetonitrile–buffer (90:10, v/v) (28 kPa). The robot placed the eluate into the evaporator (40°C) under nitrogen (207 kPa). After 1 h, the robot transferred the dried container to the balance and reconstituted the sample by adding 300 μl of mobile phase with a gas-tight syringe. The robot then vortexed the sample and injected it onto the HPLC column using the LC sip injection station with a 200-μl fixed loop.

The analytical process was serialized so that each sample was exposed and handled in an identical manner. At steady state operation of the method, one sample was being mixed and extracted, four samples were being evaporated, and one was undergoing HPLC analysis. For plasma analysis, samples were centrifuged at 325 *g* for 15 min at 4°C. This prevented fibrous material in some samples from interfering with the initial pipetting step by forcing it to the bottom and sides of the container.

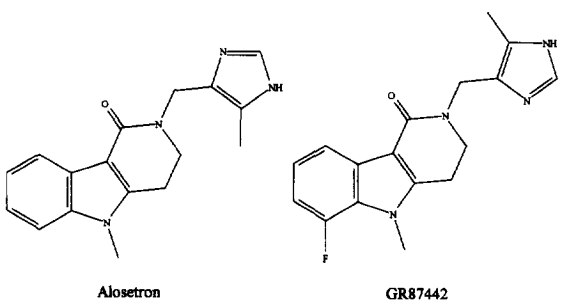

Fig. 1. Structures of alosetron (GR68755) and the internal standard, GR87442.

2.3. Chromatography

Chromatographic analysis was performed at 45°C using a Spherisorb cyanopropyl analytical column and precolumn, a mobile phase of 0.01 *M* ammonium acetate (pH 4.0)–methanol–tetrahydrofuran (70:24:6, v/v) at a flow-rate of 0.5 ml/min, and fluorescence detection (excitation at 295 nm, emission at 370 nm). The mobile phase was not recirculated during analysis.

2.4. Instrumentation

The HPLC system included a Spectroflow Model 400 solvent delivery system (Applied Biosystems, Ramsey, NJ, USA), a Model 7961 HPLC column block heater (Jones Chromatography, Littleton, CO, USA), a Model RF-551 fluorescence detector (Shimadzu, Kyoto, Japan), a 7-μm, cyanopropyl, 15 × 4.6 mm I.D. precolumn and a Spheri 5-μm, cyanopropyl, 100 × 4.6 mm I.D. analytical column (Brownlee Labs, San Jose, CA, USA). The chromatographic signal was acquired and integrated using a 3350 Laboratory Automation System (Hewlett-Packard, Avondale, PA, USA).

A Zymark Py Technology II system (Zymark Corporation, Hopkinton, MA, USA) was used to automate the process. System peripherals included a gripper hand, a 0.2–1.0 ml pipetting hand, a cold storage rack (4°C) for samples, an analytical balance (Mettler AE240, Hightstown, NJ, USA) for monitoring liquid transfers, a solid-phase extraction station, a vortex and liquid dispensing station, and a cannula sip station for making fixed loop HPLC injections. The positioning of peripheral modules about the robot is indicated in Fig. 2. As many as three gas-tight syringe hands were added to the system configuration as optional ways of adding internal standard and buffer to the samples, delivering elution reagent to the extraction columns, or for performing sample dilutions when necessary.

2.5. Validation

Calibration standards (0.1, 0.25, 0.5, 1, 3, 12 and 20 ng/ml) and quality control (QC) samples (0.3, 5 and 15 ng/ml) were prepared by mixing blank plasma or serum with aqueous solutions of alosetron. The initial stock solutions of alosetron and GR87442 were prepared in 20% methanol at 1 mg/ml. Serial dilutions were prepared daily when using a water diluent instead of buffer or partial organic solvent solutions due to adsorption difficulties with some types of glass. The calibration standards were prepared either immediately prior to each assay run or in a batch and stored frozen (−20°C) until required. The QC samples were prepared in a batch and stored frozen.

Formal method validation comprised four analytical runs of 50–99 samples, testing the full sample capacity of the system. Calibration standards were analyzed in duplicate with one set placed at the beginning of each run and a second set placed at the end. QC samples were analyzed in duplicate and interspersed throughout the analytical run. Additional samples were analyzed to assess intra-day variability, sample dilution capability, and stability. Metabolites, potential concomitant medications, and different plasma and serum lots were analyzed to assess assay specificity. Extraction efficiency was measured at all three QC concentrations by peak-height comparison with duplicate, spiked mobile phase samples.

During study sample analysis, the robotic system continuously validated itself. The robotic system kept track of sample and container parameters, and monitored for handling errors by means of mechanical switches, optical switches and electronic resistance sensory ability. All liquid transfers were gravimetrically confirmed and reported by printer output. The internal standard provided additional confirmation of sample preparation consistency.

3. Results and discussion

3.1. Formal validation

The method was selective for alosetron and GR87442 with respect to endogenous plasma and serum components, identified human metabolites, and those potentially co-administered compounds listed in Table 1. Alosetron is extensively metabolized in humans. The major metabolite (N-demethylated alosetron) is excreted in the urine as a glucuronide conjugate. Retention times of alosetron and GR87442 were approximately 10.1 and 13.7 min, respectively. Examples of extracted plasma chromatograms generated during the validation are shown in Figs. 3–5.

The method was validated for the quantitation of alosetron plasma concentrations from 0.1 to 20 ng/ml with and without an internal standard. Linear regression was performed using either peak-height ratios or peak heights, both with a $1/x^2$ ($1/\text{concentration}^2$) weighting. The mean correlation coefficient was 0.9984 using an internal standard and 0.9970 without an internal standard. Further discus-

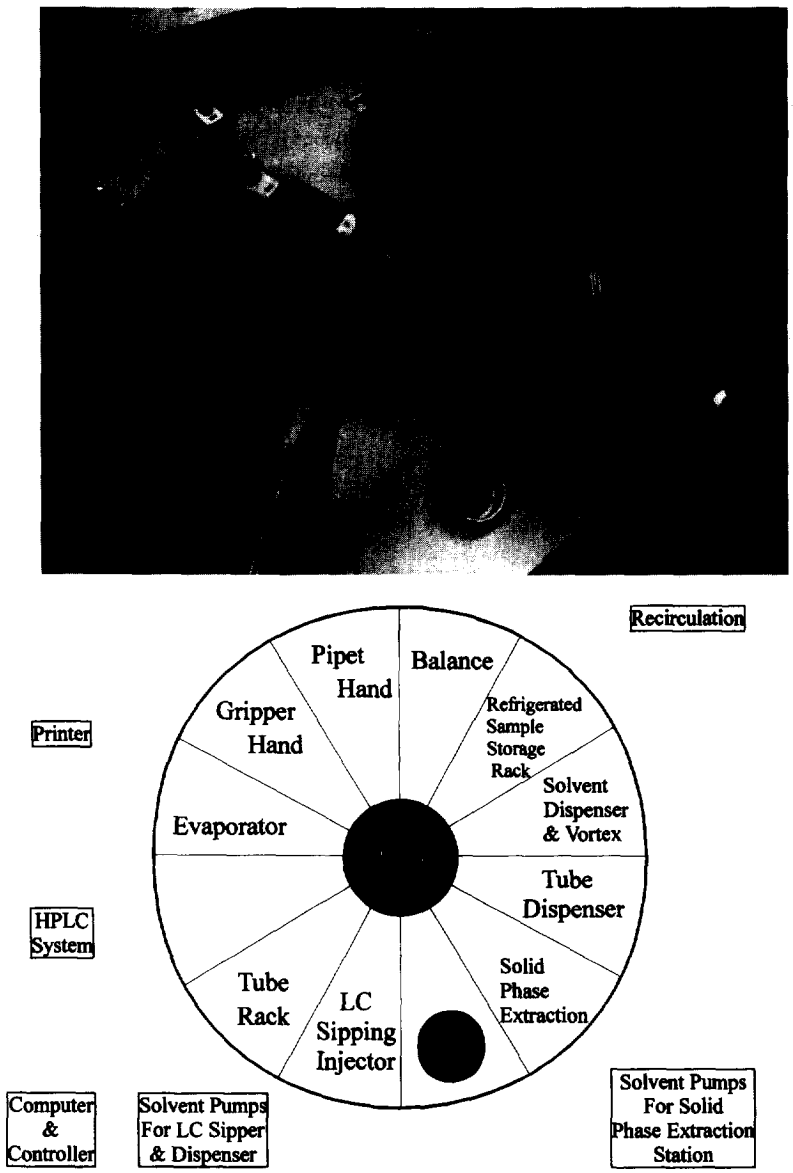

Fig. 2. Robotic system configuration for alosetron assay.

sion will center around internal standard curve results. The mean regression equation was $y = 0.148x + 0.012$ where y is the peak-height ratio (alosetron/GR87442).

Intra-day mean accuracy was within 4% of the theoretical concentration for all standard and QC concentrations except at the lower limit of quantitation (LLOQ), 0.1 ng/ml, where the difference was 10%. Precision for within-day QC concentrations yielded coefficients of variation < 2% and was 7% at the LLOQ (0.1 ng/ml). Mean accuracy for between-day standards and quality control concentration levels was within 5% of the nominal standard and QC concentrations (Table 2). The between-day coefficients of variation for all standard and QC concentrations ranged from 1 to 9% (Table 2). The extraction efficiency for alosetron ranged from 91 to 95% throughout the range of the curve with a mean

Table 1
Potentially co-administered compounds screened for assay interference

Amitriptyline	Diazepam	Methotrexate
Carbamazepine	Digoxin	Phenobarbital
Carmustine	Etoposide	Phenytoin
Chlorpromazine	Furosemide	Propranolol
Cimetidine	Halperidol	Ranitidine
Cisplatin	Ibuprofen	Theophylline
Cyclophosphamide	Imipramine	Triazolam
Dexamethasone	Indomethacin	Warfarin

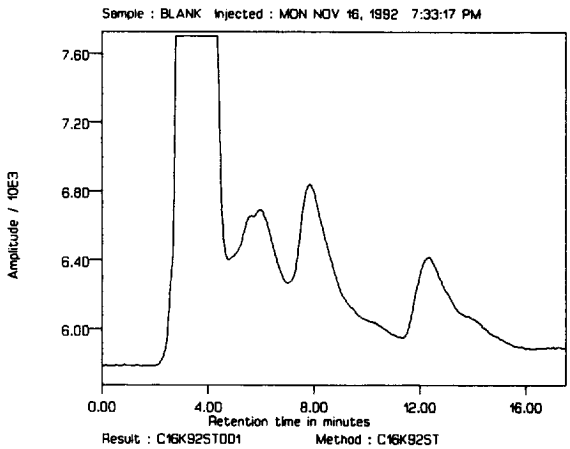

Fig. 3. Chromatogram of blank human plasma extract.

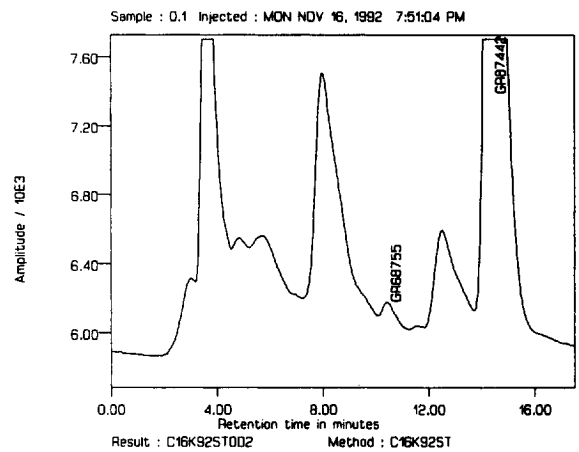

Fig. 4. Chromatogram of 0.1 ng/ml alosetron standard human plasma extract.

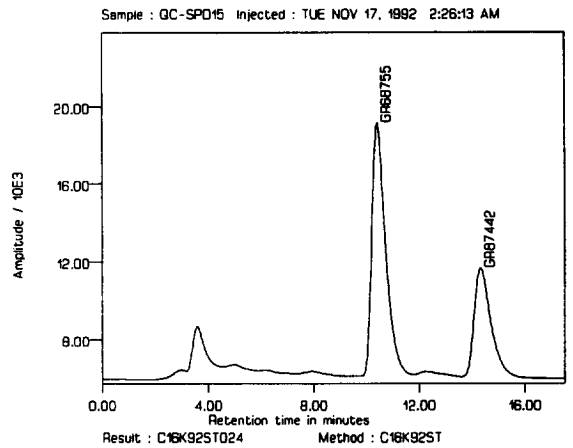

Fig. 5. Chromatogram of 15 ng/ml alosetron QC human plasma extract.

recovery of 93%. The extraction efficiency for the internal standard was 99% at the concentration used (10 ng/ml).

3.2. Ruggedness

The method was originally developed using robotic sample preparation which afforded control and validation of experimental parameters. An experimental design strategy, using RS1 Explore/Discover software (BBN, Cambridge, MA USA), guided method optimization and ruggedness assessment for the sample preparation [3,4]. A central composite, fractional factorial design was employed to model parameters of the solid-phase extraction. This approach afforded rapid elucidation of a dual retention mechanism, which led to a 100% improvement in the extraction efficiency of the method. The assay was initially validated for a concentration range of 1 to 250 ng/ml in order to support early dose ranging studies, later revalidated for 0.25 to 50 ng/ml and currently is validated for 0.1 to 20 ng/ml in support of increasingly lower clinical doses. It has also been cross-validated for use by different analysts, different robots, manual sample processing and different countries.

The method has been operated with several different extraction column sorbent lots. It is not sensitive to moderate variation of buffer strength and pH for the solid-phase extraction reagents, but retention

Table 2
Inter-day precision and accuracy of the assay for alosetron in plasma

Analysis day	Predicted concentration of alosetron in plasma (n=8) (ng/ml)									
	Quality controls			Standards						
	0.3	5	15	0.1	0.25	0.5	1	3	12	20
1	0.30	5.11	15.55	0.10	0.23	0.45	0.97	2.92	12.42	21.23
	0.27	5.25	16.00	0.11	0.24	0.49	1.01	3.03	12.57	21.34
2	0.31	4.76	14.65	0.09	0.24	0.52	1.00	2.97	11.96	20.52
	0.26	4.91	14.87	0.12	0.25	0.50	0.98	2.91	12.05	20.68
3	0.31	5.28	15.15	0.10	0.23	0.49	0.99	2.79	12.29	20.82
	0.28	5.07	15.24	0.10	0.25	0.48	1.00	3.08	12.43	21.25
4	0.29	4.88	15.21	0.10	0.25	0.48	0.98	3.05	12.42	20.18
	0.27	5.03	14.73	0.10	0.25	0.49	0.97	2.98	12.29	20.37
Mean	0.29	5.04	15.18	0.10	0.24	0.49	0.99	2.97	12.30	20.80
Nominal	95.42	100.73	101.17	102.50	97.00	97.50	98.75	98.88	102.53	103.99
C.V. (%)	6.72	3.59	2.94	8.65	3.66	4.07	1.51	3.14	1.67	2.10

times can be shifted by variation of these parameters in the mobile phase. The method has also been operated with different analytical column production lots and brands including CPS Hypersil material from Keystone (Bellefonte, PA, USA). Substantial column lot variability has been observed more recently, reflecting a silanol interaction effect on the retention mechanism of the method. The extracted plasma chromatogram from a clinical patient (Fig. 6) illustrates this point when comparing compound retention times with those in Figs. 3–5. It also provides a worst case example of an endogenous peak found to varying degrees in some subjects which required maintaining considerable separation between the compounds of interest. Small adjustments in the percentage of organic solvent used in the mobile phase have served to address this column lot variability. The HPLC separation time was the rate-limiting step for the serialized robotic processing.

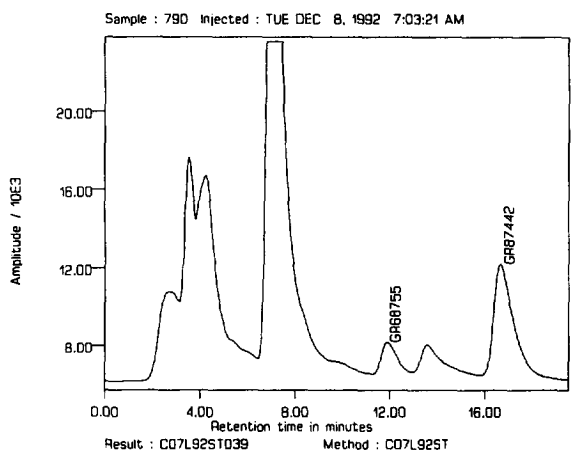

Fig. 6. Chromatogram of a 6-h post-oral 1 mg dose patient sample.

Acceptable stability for alosetron in human serum, within 10% of original concentrations, has been observed in samples stored at $\leq -20°C$ for at least two years. Sample stability was also confirmed through three daily freeze-thaw cycles. Samples exposed to either ambient or refrigerated storage were stable at least 72 h. Degradation of the internal standard, GR87442, was observed during method development under prolonged exposure to low wavelength UV radiation from unshielded, fluorescent laboratory lighting. The problem was addressed in various laboratories by either installing plastic covers over the fluorescent lights or covering the internal standard reservoir from light.

3.3. Application

A sensitive and selective method for the determination of alosetron in serum or plasma was necessary to support human pharmacokinetic/biopharmaceutic studies. An example is presented where the method was used to measure alosetron plasma concentrations in a pharmacokinetic study designed to determine the disposition of alosetron in humans [5]. A representative plot of alosetron plasma concentration versus time after 1 mg oral administration of alosetron for

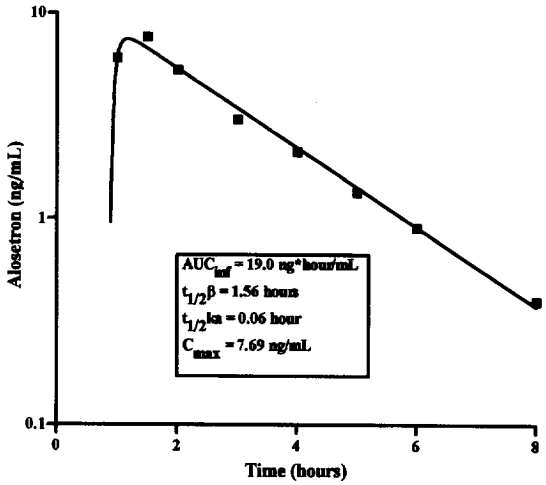

Fig. 7. Plasma concentration–time profiles of alosetron for one patient. The symbols are actual data and the solid line represents the theoretical line obtained by the curve-fitting procedure.

one patient is shown in Fig. 7. The fitting of these concentrations to a one-compartment model with oral absorption gave values of 0.06 h (or 3.6 min) for the absorption half-life and 1.56 h for the terminal elimination half-life while area under the plasma concentration–time curve was 19.0 ng · h/ml.

The use of a fully automated system for the determination of alosetron in serum or plasma enhanced the analytical precision, efficiency and safety. The statistical improvement in analytical precision achieved by using a robotic system for solid-phase extraction (with serial sample analysis and positive pressure processing of solvents) compared with manual solid-phase extraction (batch analysis and vacuum box processing of solvents) has been demonstrated [6]. In addition, a customized, variable transducer control of the positive pressure applied to a solid-phase extraction column was introduced. This new device allowed for a different pressure setting after each solvent addition, offering fine adjustments to the extraction chemistry without compromising precision.

The efficiency of a fully automated system was repeatedly demonstrated during the analysis of over 7000 clinical study samples in the United States alone. The robot routinely analyzed 100 samples every 24 h while the analyst processed and documented data from the previous day. Automation enhanced analyst safety by limiting manual handling of human biological fluid samples to loading the original sample containers into the refrigerated rack. This method has also been applied for the primary analysis of other 5-HT_3 antagonists such as ondansetron and GR87442 in serum and urine with small variation of the HPLC conditions.

In conclusion, the method described is specific, accurate and rugged. The fully automated version of the assay offers improved analytical precision, efficiency and safety. The method is suitable for use in support of human pharmacokinetic/biopharmaceutic studies.

Acknowledgments

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