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Automated high-performance liquid chromatographic method for the determination of a neuraminidase inhibitor (GG167) in human serum by pre-column fluorescence derivatisation using benzoin

R.J. Stubbs*, A.J. Harker

Drug Metabolism I, Glaxo Research and Development Ltd, Greenford Road, Greenford, Middlesex UB6 0HE, UK

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Abstract

A pre-column fluorescence derivatisation high-performance liquid chromatographic method for the analysis of a neuraminidase inhibitor, GG167, in human serum is described. GG167 was extracted from serum samples using Bond Elut SCX solid-phase extraction cartridges, followed by derivatisation with benzoin prior to reversed-phase chromatography with fluorescence detection. This method has been automated using a Zymark robot and used in the analysis of human serum samples from clinical studies. The method has been shown to be valid over a concentration range of 10–800 ng/ml using a 1-ml sample volume.

1. Introduction

GG167 (5-acetylamino-4-guanidino-2,6-anhydro-3,4,5-trideoxy-D-glycero-D-galacto-non-2-enoic acid) (I) is a potent and selective inhibitor of influenza viral neuraminidase [1], an enzyme responsible for the release of new virus particles from infected cells. Compound I is extremely effective when administered intranasally in animal models of infection (mice and ferrets), with doses as low as 50 μg/kg bd showing good effect. The drug is currently being developed for the treatment and prophylaxis of influenza A and B.

A sensitive analytical method was required for the determination of I in human serum for the monitoring of clinical studies and for obtaining pharmacokinetic data in man. This information could not be obtained using an existing HPLC method which was based on protein precipitation, for sample preparation and UV detection. The sensitivity of this method was limited by the poor UV absorptive properties of I, thus the method could only be used for the monitoring of concentrations of I in serum from toxicity studies.

Fluorescence derivatisation was investigated due to the high sensitivity which this can provide [2]. Examples of fluorescence derivatisation of guanidino compounds using benzoin have previously been reported which allow the detection of concentrations in the low ng/ml region [3–5]. The aim of this work was to develop a sensitive method for the determination of I in human

* Corresponding author.

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serum which could be fully automated using a Zymark robot.

2. Experimental

2.1. Materials and reagents

Compound I (Fig. 1) was synthesised by Glaxo Research and Development (Greenford, UK). Methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased from Rathburn (Walkerburn, UK). Triethylamine (Sequanal grade) was purchased from Pierce and Warriner (Chester, UK). Formic acid (AR), ethylene glycol (GPR), potassium hydroxide (KOH) (AnalaR) and Tris(hydroxymethyl)methylamine (AnalaR) were purchased from BDH (Dagenham, UK).

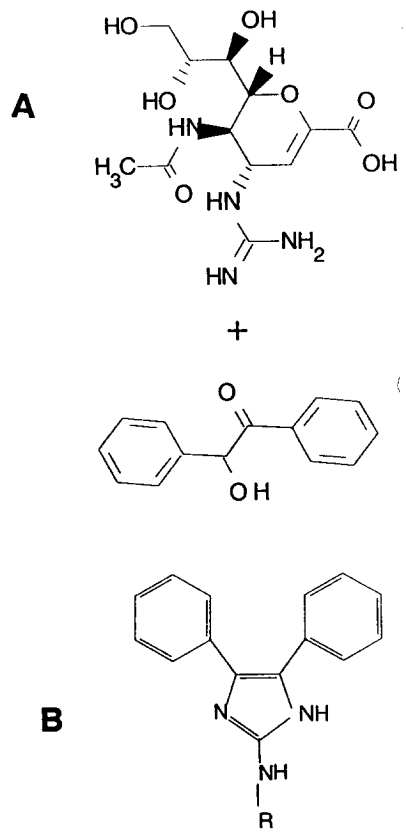

Fig. 1. Proposed reaction of (A) I and benzoin to give the product (B).

Trifluoroacetic acid was purchased from Sigma (Poole, UK). Benzoin, β-mercaptoethanol and sodium sulphite were purchased from Aldrich (Gillingham, UK). Hydrochloric acid was purchased from Fisons (Loughborough, UK).

Benzoin (21.25 mg) was dissolved in 25 ml of ethylene glycol to obtain a 4.0 m*M* benzoin solution.

β-Mercaptoethanol (750 μl), sodium sulphite (2.52 g) and 5 *M* KOH (10 ml) were diluted to 100 ml with distilled water to obtain a β-mercaptoethanol (0.1 *M*)–sodium sulphite (0.2 *M*)–KOH solution (0.5 *M*).

1 *M* Tris(hydroxymethyl)methylamine (100 ml) and 1 *M* hydrochloric acid (29.4 ml) were added to distilled water and made up to a final volume of 2 l to obtain the Tris–hydrochloric acid buffer (0.05 *M*, pH 8.5).

2.2. Preparation of stock solutions of I

For preparation of samples to be used in the validation of the assay I was weighed out in duplicate and dissolved in distilled water to give two solutions designated A and B. Dilutions of each stock were made with distilled water as appropriate, including 10 μg/ml and 1.0 μg/ml solutions. Dilutions of stock A were used in the preparation of the calibration standards and dilutions of stock B were used in the preparation of the validation and quality control samples (QCs).

2.3. Preparation of calibration standards and QCs

Control human serum was spiked with aqueous solutions of I derived from stock A to provide standards in the range of 5–800 ng/ml. Serum QC samples were prepared by spiking control serum with stock B solutions to provide low, medium and high concentrations (30, 120 and 320 ng/ml, respectively) of I for the determination of inter-assay performance. Serum was also spiked from stock B to provide samples within the calibration range for the determination of intra-assay performance.

2.4. Extraction procedure

An aliquot of serum (1 ml) was mixed with an equal volume of 10% formic acid. This mixture was applied to a SCX Bond Elut which had been activated with 2 ml methanol and 2 ml 10% formic acid. The cartridge was then washed sequentially with 2 ml 1% trifluoroacetic acid in methanol and 2 ml distilled water. Finally the cartridge was eluted with 4×0.5 ml 10% triethylamine in methanol–water (50:50) which was collected into a clean tube and evaporated to dryness at 70°C under a gentle stream of nitrogen.

2.5. Fluorescence derivatisation

Derivatisation of I was performed by adding 75 μl of benzoin solution and 150 μl of β-mercaptoethanol–sodium sulphite–KOH solution to the extraction residue obtained from the serum extract. This was vortex-mixed and then incubated for 3 min at 100°C prior to injecting a 100 μl aliquot onto the HPLC column.

2.6. Chromatographic analysis

The chromatographic analyses were performed using an HPLC system consisting of a Constametric 4100 solvent delivery system with membrane degasser (LDC, Staffordshire, UK) and a variable wavelength spectrofluorimetric detector (Model RF-551 from Shimadzu, Kyoto, Japan). Integration of chromatograms was performed using a Multichrom data system (VG, Manchester, UK).

Preparation of samples was automated using a Zymate II System (Zymark, Hopkinton, MA, USA). The system was equipped with the following PySections: General Purpose Hand, Internal Standard Hand, Rack, Test Tube Dispenser, Dilute and Dissolve, three Master Laboratory Stations, Liquid/Solid Extraction, Evaporation, Heater, Disposal and LC Sipping Injection. A 100×4.6 mm I.D. ODS Hypersil (Capital HPLC, West Lothian, UK) analytical column was eluted with a mobile phase of 20% acetonitrile–80% Tris–HCl buffer (pH 8.5, 0.05 *M*, Aq) at a flow-rate of 1 ml/min. After 12 min the mobile phase was changed to 80% acetonitrile–20% Tris–HCl buffer for 5 min to remove late eluting peaks which otherwise necessitated a run time of 1 h and 20 min, the system was then re-equilibrated for 10 min at the initial conditions prior to the next injection, giving a total run time of 27 min. The fluorescence of the column eluent was monitored using an excitation wavelength of 325 nm and an emission wavelength of 442 nm.

2.7. Intra-assay precision and bias

A set of calibration standards prepared from stock A solutions and six-fold replicates of serum spiked from stock B at the same concentration as six of the calibration standards, were analysed as a single batch using the methods described. The intra-assay variability of the method was determined using the coefficient of variation of replicate assays ($n = 6$) for each of the six selected concentrations on a single occasion.

2.8. Inter-assay precision and bias

On five separate occasions duplicate quality control samples at three concentrations were assayed alongside additional samples and a set of calibration standards. The inter-assay precision was determined as the coefficient of variation for each set of QC samples ($n = 10$). On each occasion samples were added to the batch in order to make it representative in size of a standard analytical batch.

2.9. Relative recovery

The relative recovery of I from serum was determined by comparing the slope of the calibration line obtained from aqueous non-extracted solutions of I, derivatised and injected onto the analytical column, with the slope of the calibration line obtained from extracted samples. Recovery was determined over the range 5–800 ng/ml.

2.10. Specificity

Samples of human serum taken from twelve subjects were tested to determine whether endogenous components would interfere with the analysis.

3. Results and discussion

The proposed reaction mechanism is illustrated in Fig. 1, where (A) illustrates I and benzoin, respectively, and (B) shows the proposed (fluorescent) product. The optimum reaction time was found to be 3 min; this was a compromise between product formation and its subsequent degradation under the conditions used.

The initial method for benzoin derivatisation incorporated the addition of acid to the incubation mixture after a fixed time. The addition of acid served two purposes: it stopped the reaction and it stabilised the derivatised product.

When the acidification step was used in the procedure for derivatising I, a fine precipitate was produced. Formation of such a precipitate was very undesirable in an assay which was intended for routine analysis of large numbers of samples. Without centrifugation of all samples there was a high possibility that some precipitated material would be injected onto the analytical column and this could lead to a non-robust method.

The need to acidify then centrifuge the samples was avoided by the use of a Zymark robot to process samples. The robot was able to perform tasks to precise (timed) intervals thereby removing the need to stop the reaction.

After investigation of various reaction conditions a method was arrived at requiring the manipulation of only two reagents. The conditions were optimised so as to minimise the number of steps in the derivatisation procedure. The final method used benzoin in ethylene glycol as one reagent and β-mercaptoethanol, sodium sulphite and KOH pooled as the second reagent.

Dimethylformamide and dimethylsulphoxide were investigated as alternative solvents for use with benzoin, but neither offered an advantage over ethylene glycol.

The product yield was found to increase with increasing benzoin concentration up to 4.0 m*M*, which was the limit of the benzoin solubility in ethylene glycol.

The response of peak height versus concentration was linear over the calibration range used and $1/x^2$ weighted regression was used in constructing the calibration lines. A typical calibration line is presented in Fig. 2.

The method displayed good inter- and intra-assay precision, with little bias, over the validated concentration range (Tables 1 and 2). The limit of quantification for the method was taken to be 10 ng/ml, since this was the lowest calibration standard used that gave an acceptable coefficient of variation (2.3%). The 5 ng/ml standard could not be consistently quantified and had a C.V. of 49.4%.

The recovery investigations comparing the slopes of the calibration lines obtained from the aqueous and serum standards gave a mean recovery of 87.5%. No differences were observed in the recoveries across the entire calibration range.

Blank sera showed no endogenous interference in the region of the chromatogram where derivatised I elutes as can be seen in Figs. 3, 4 and 5 which are sample chromatograms of blank serum, 50 ng/ml serum standard and a clinical sample taken 3 h after an inhaled dose, respectively.

The automated method was subsequently used to determine the pharmacokinetics of I in man following single intravenous (i.v.) infusions of 1, 2, 4, 8 and 16 mg, single and multiple (six times daily for five days) intranasal (i.n.) doses of 4, 8 and 16 mg and single doses of 4, 8 and 16 mg administered by nebuliser (i.h.). Eight healthy male subjects participated in the i.v., twenty four in the i.n. and twenty in the i.h. administration studies. The kinetics of I were linear in the dose range studied for all administration routes and they were not modified after repeated adminstration [6].

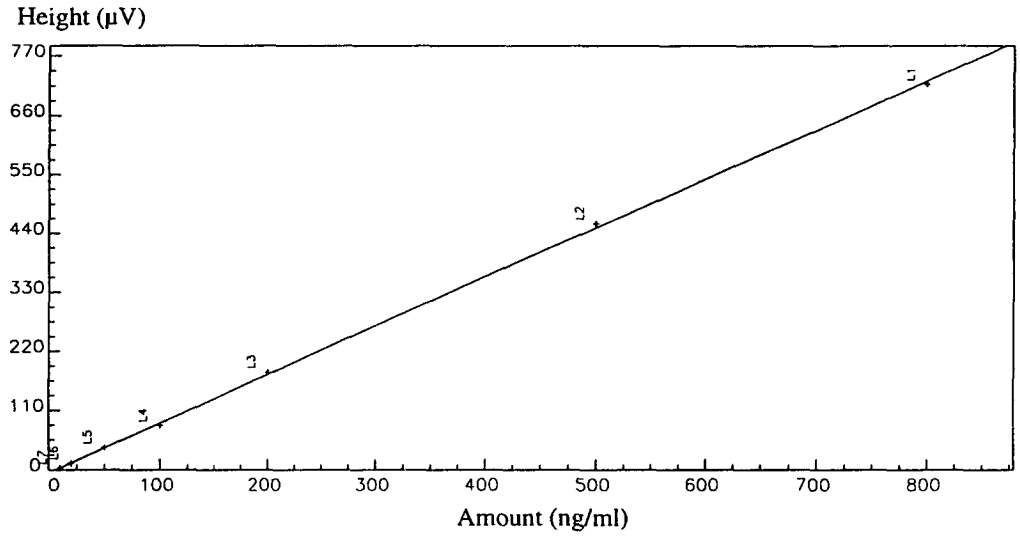

Fig. 2. Typical calibration line.

Table 1
Intra-assay variation ($n = 6$)

Theoretical concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Bias (%)	C.V. (%)
5	6.5 ± 3.2	+29.3	49.4
10	9.3 ± 0.2	−7.2	2.3
20	16.7 ± 0.5	−16.4	3.1
100	87.8 ± 3.2	−12.2	3.7
200	180.0 ± 4.3	−10.0	2.4
800	773.0 ± 17.2	−3.4	2.2

4. Conclusions

Compound I was extracted from serum samples using Bond Elut SCX solid-phase extraction cartridges, followed by derivatisation with benzoin prior to reversed-phase chromatography with fluorescence detection. An HPLC-fluorescence method for the determination of I in

Table 2
Inter-assay variation ($n = 10$)

Theoretical concentration (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Bias (%)	C.V. (%)
30	26.7 ± 0.99	−11	3.7
120	120 ± 13.0	0	10.8
320	324 ± 16.5	+1.3	5.1

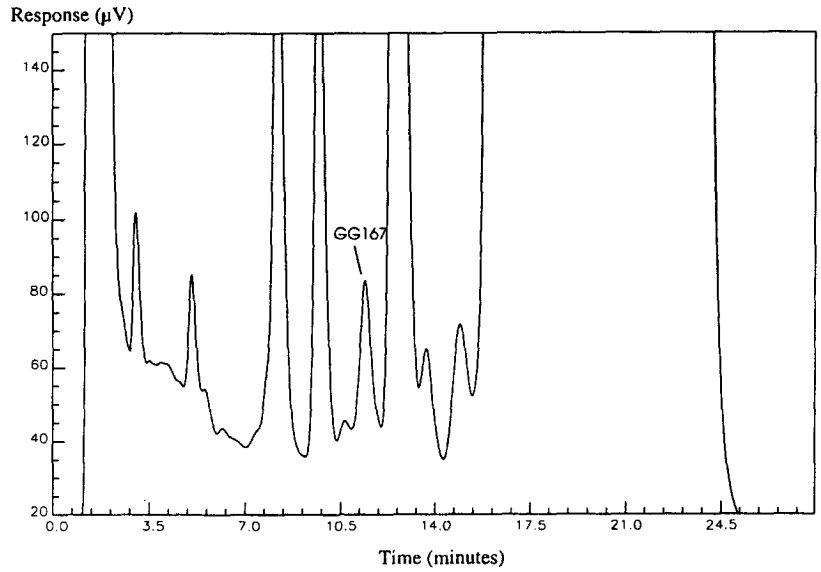

Fig. 3. Sample chromatogram of serum containing I at 50 ng/ml.

human serum has been successfully validated as an automated process using a Zymark robot. The method has been shown to be valid over a concentration range of 10–800 ng/ml using a 1-ml sample volume.

The analysis of serum samples by this method showed it to be both sensitive and robust. The method is currently being used for the analysis of samples from clinical studies as part of the ongoing development programme for I.

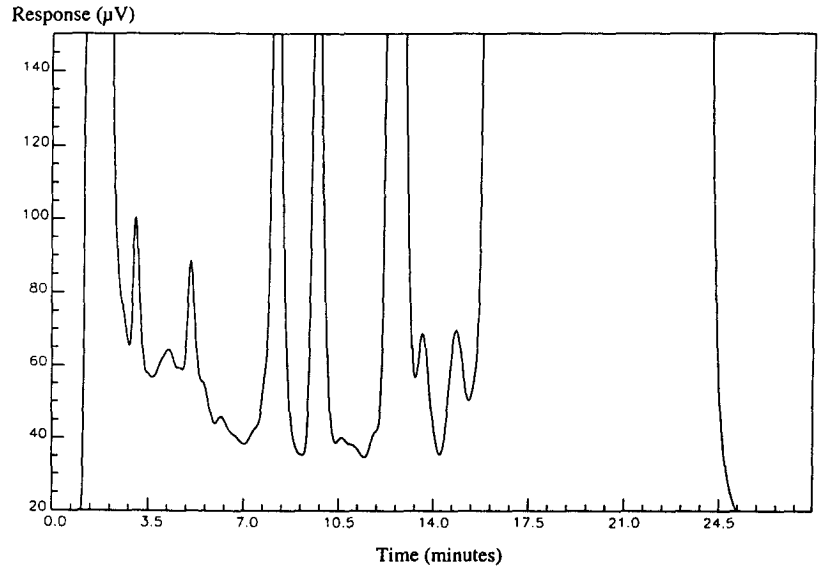

Fig. 4. Sample chromatogram of blank serum.

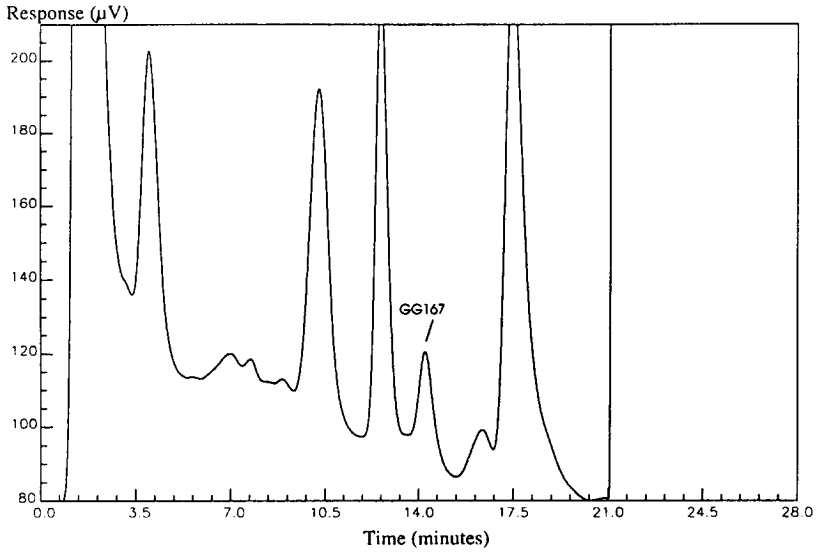

Fig. 5. Chromatogram of a serum sample taken 3 h after a 10 mg inhaled dose of I (37 ng/ml I).

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