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Determination of a novel growth hormone secretagogue (MK-677) in human plasma at picogram levels by liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry

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Abstract

A sensitive and specific method for the determination of *N*-[1(*R*){[1,2-dihydro-1-methylsulfonylspiro(3H-indole-3,4′-piperidin)-1′-yl]carbonyl}-2-(phenylmethoxy)ethyl]-2-amino-2-methylpropanamide (MK-677, I), a growth hormone secretagogue, has been developed. The method is based on high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS–MS) detection. The analyte and internal standard (II) were isolated from the basified plasma using a liquid–liquid extraction with methyl-*tert.*-butyl ether (MTBE). The organic extract was evaporated to dryness, the residue was reconstituted in mobile phase and injected into the HPLC system. The MS–MS detection was performed on a PE Sciex API III Plus tandem mass spectrometer using a heated nebulizer interface. Multiple reaction monitoring of parent→product ion combinations at *m/z* 529→267 and 527→267 was used to quantify I and II, respectively. The assay was validated in human plasma in the concentration range of 0.1 to 100 ng/ml, and the limit of quantification (LOQ) was 0.1 ng/ml. The precision of the assay, as expressed as coefficients of variation (C.V.,%) was less than 7% at all concentrations within the standard curve range, with adequate assay specificity and accuracy. The HPLC–MS–MS method provided sufficient sensitivity to completely map the pharmacokinetic time-course following a single 5-mg oral dose of I.

Keywords: Growth hormone secretagogue; MK-677

1. Introduction

Interest in growth hormone (GH) secretagogues has intensified in the past several years based on promising applications of recombinant GH in animals and humans. The long term metabolic effects of GH include the stimulation of muscle protein synthesis, lipolysis and an inhibition of glucose uptake and utilization. However, the utility of recombinant GH is limited by the need to administer by injection and some side effects reported during treatment [1]. Therefore, it is speculated that GH secretagogues may offer a physiological advantage over recombinant GH therapy by providing a more sustained release of GH [2]. In addition to the GH-releasing peptide [3,4], a nonpeptide GH secretagogue (L-692,429) has been recently discovered [5]. This nonpeptide analog has been shown to elevate GH in young male volunteers and in healthy older subjects [6,7], but the limited oral bioavailability inhibits the feasibility of its use as a therapeutic agent [6].

N - [1(*R*){[1,2-Dihydro-1-methylsulfonylspiro(3H-

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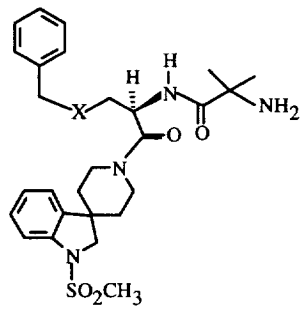

I; X = O
II; X = CH_2

Fig. 1. Chemical structures of I and an internal standard II.

indole - 3,4′ - piperidin) - 1′ - yl]carbonyl} - 2 - (phenylmethoxy)ethyl] - 2 - amino - 2 - methylpropanamide (MK-677, I, Fig. 1) is a newly discovered GH secretagogue, currently under investigation at Merck Research Laboratories [8]. Its superior oral potency and duration of action led to safety assessment studies and to ongoing clinical trials. In order to provide bioanalytical support for pharmacokinetic evaluation of I at low therapeutic doses, an analytical method with a limit of quantification (LOQ) of less than 1 ng/ ml of plasma was required.

Previously, naphthalene-2,3-dicarboxyaldehyde (NDA) had been used in our laboratories to derivatize a primary amine containing peptide [9] and other drug candidates containing a primary amino group [10,11] to a highly fluorescent benzo[*f*]isoindole derivatives. Since I contains a primary amino group, initially, derivatization of I with NDA in the presence of thiol nucleophiles, in a manner similar to [9–11] was attempted. The efficiency of derivatization was poor probably due to the steric hindrance for the reagent in approaching an amino group attached to the tertiary carbon atom. Instead, an HPLC method with pre-column chemical derivatization of the primary amino group of I with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was developed [12]. The AQC reagent forms fluorescent derivatives with both primary and secondary amines, and steric requirements are probably less severe in comparison with NDA. The limit of quantification (LOQ) of the AQC derivatization method was 0.5 ng/ml which was insufficient to determine plasma concentrations 24 h after oral administration of I. In addition the method required an extensive, multi-step sample preparation procedure, chromatography with a column-switching system and a relatively long HPLC analysis time (26 min). Therefore, in order to support large scale human pharmacokinetic studies with I, a more efficient and sensitive method was desirable. Since the combination of HPLC with atmospheric-pressure chemical ionization (APCI) tandem mass spectrometry (MS–MS) was shown to provide a convenient and highly efficient method for the quantitation of drugs in biological fluids (for a number of recent examples from the authors' laboratory see Refs. [13–16]), the feasibility of utilization of HPLC–MS–MS for quantification of I was evaluated. Using this technique, an assay for I in human plasma with the LOQ of 0.1 ng/ml and a short runtime of 6 min was developed. The details of the HPLC–MS–MS methodology and its application to support pharmacokinetic studies in humans after low (5 mg) oral doses of I are the subject of this paper.

2. Experimental

2.1. Materials

Compound I was synthesized as a methanesulfonate salt at the Medicinal Chemistry Department of Merck Research Laboratories (Rahway, NJ, USA). All solvents and reagents were of HPLC or analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The drug free human heparinized plasma originated from Biological Specialties (Lansdale, PA, USA). Air (hydrocarbon-free), nitrogen (99.999%) and argon (99.999%) were purchased from West Point Supply (West Point, PA, USA).

2.2. Instrumentation

A Perkin-Elmer (PE) Sciex (Thornhill, Ontario, Canada) API III+ tandem mass spectrometer equipped with heated nebulizer interface, a Waters

Associates (Waters-Millipore, Milford, MA, USA) WISP 715 autoinjector and PE biocompatable binary pump (Model 250) were used for all HPLC–MS–MS analyses. The data were processed on a MacIntosh Quadra 900 microcomputer.

2.3. Chromatographic conditions

HPLC separation was performed on a C_{18} BDS 150×4.6 mm I.D., 3-μm analytical column (Keystone Scientific, Bellefonte, PA, USA) coupled with a 2 μm in-line filter. The mobile phase was a mixture of 50% acetonitrile and 50% water (v/v) containing 0.1% formic acid and 10 m*M* ammonium acetate, and was delivered at a flow-rate of 1.0 ml/min. The retention times for I and II were 3.5 and 4.5 min, respectively.

2.4. HPLC–MS–MS conditions

A PE Sciex triple quadrupole mass spectrometer was interfaced via a Sciex heated nebulizer probe to HPLC system and gas phase chemical ionization was effected by a corona discharge needle (+4 μA) using positive ion APCI. The heated nebulizer probe was maintained at 500°C. The nebulizing gas (air) pressure and auxiliary flow were set at 550 kPa and 2.0 l/min, respectively. Curtain gas flow (nitrogen) was 0.9 l/min and the sampling orifice potential was set at +40 V. The dwell time was 400 ms, and the temperature of the interface heater was set at 60°C. The mass spectrometer was programmed to admit the protonated molecular ions $[M+H]^+$ at *m/z* 529 (I) and *m/z* 527 (II) via the first quadrupole filter (Q1), with collision-induced fragmentation at Q2 (collision gas argon, 280×10^{13} atoms cm^{-2}), and monitoring the product ions via Q3 at *m/z* 267 for both I and II. The electron multiplier setting was −4.2 kV. Peak-area ratios obtained from multiple reaction monitoring of the analyte (*m/z* 529→267) to II (*m/z* 527→267) were utilized for the construction of calibration curves, using weighted ($1/y^2$) linear least square regression of the plasma concentrations and the measured area ratios. Data collection, peak integration and calculations were performed using MacQuan PE Sciex software.

2.5. Standard solutions

A stock solution of I (1 mg/ml) was prepared in methanol. This solution was further diluted with methanol to give a series of working standards with the concentrations of 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 50.0 and 100.0 ng/ml. The internal standard II was also prepared as a stock solution (1 mg/ml) in methanol by dissolving 10 mg of solid II in 10 ml of methanol. A working standard of 10 ng/ml was prepared by serial dilutions of the stock standard with methanol, and was used for all analyses. All standards were prepared once a month and stored at 5°C.

A series of quality control (QC) samples in plasma at concentrations of 1 and 75 ng/ml were also prepared. Aliquots (1.25 ml) of these solutions were placed in 2-ml plastic tubes, stored at −20°C, and analyzed daily with clinical samples. The calculated concentrations of the QC samples were compared on a day-to-day basis to assess the inter-day assay performance.

2.6. Sample preparation

A 1-ml aliquot of plasma was pipetted into a 15-ml centrifuge tube and 100 μl of the working standard solution of II (equivalent to 10 ng/ml of II) was added, followed by the addition of 1 ml of 0.2 *M* carbonate buffer (pH 9.8), and 7 ml of methyl-*tert.*-butyl ether. After capping tubes with PTFE-lined caps, the mixture was rotate-mixed for 15 min, the tubes were centrifuged and the organic layer was transferred to clean centrifuge tube. The organic extract was evaporated to dryness under a stream of nitrogen at 50°C, the residue was reconstituted in 750 μl of the mobile phase and a 150-μl aliquot was injected onto the HPLC–MS–MS system.

2.7. Precision, accuracy, recovery and specificity

The precision of the method was determined by the replicate analyses ($n=5$) of human plasma containing I at all concentrations utilized for constructing calibration curves. The linearity of each standard curve was confirmed by plotting the peak-area ratio of the drug to I.S. versus drug concentration. The

unknown sample concentrations were calculated from the equation $y=mx+b$, as determined by weighted ($1/y^2$) linear regression of the standard line. The standard curve was prepared and assayed daily with quality control and unknown samples. The accuracy of the method was expressed by [(mean observed concentration)/(spiked concentration)]× 100. Assay specificity was assessed by running blank control and patients' pre-dose biological fluid samples. No endogenous interferences were observed. The recovery was determined by comparing the peak area of I extracted from biological fluids to that of standards injected directly.

3. Results and discussion

The positive ion mass spectra (Q1) of I and II indicated the presence of predominantly the protonated molecules $[M+H]^+$ of these compounds at m/z 527 and 529, respectively. The MS–MS product mass spectra of these protonated molecules showed the presence of intense product ions at m/z 267 for both analytes (Fig. 2).

Multiple reaction monitoring using the parent→product ion combinations at m/z 529→267 for I and m/z 527→267 for II allowed highly sensitive detection of the analytes.

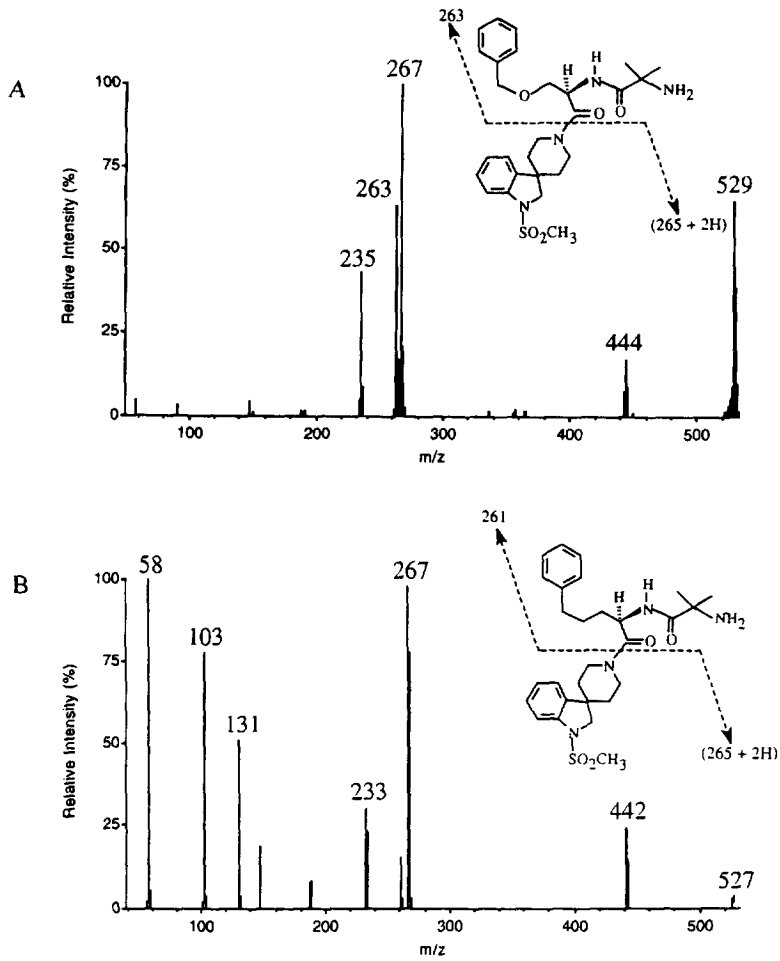

Fig. 2. Product mass spectra of the protonated molecules of I (m/z 529, A) and the internal standard (II, m/z 527, B).

The isolation of I and II from plasma was based on a simple one step liquid–liquid extraction from basified plasma, evaporation of the extract to dryness, reconstitution of the residue in the mobile phase and injection into the HPLC system. Since the pK_a of I is 7.8, the pH of plasma before extraction was adjusted to pH 9.8 to maximize the recovery of the drug. By minimizing the number of extraction steps, the adsorption problems commonly associated with the isolation of compounds containing an amino group were eliminated as indicated by high recovery of the drug. The mean recovery of I from plasma was 97±4% over the entire standard curve range (n=9) in two different lots of plasma.

Separation of I from II and from matrix interferences potentially affecting ionization of the analytes in the APCI source was achieved on a relatively long (150×4.6 mm I.D.), 3 μm analytical column. Under these conditions, the retention factors (k) for I and II were about 1.5 and 2.1, respectively. In order to assess the matrix effect on the efficiency of ionization and peak areas of the analytes, both I and II were spiked into plasma extracts originating from different subjects and from different sources. Practically the same peak areas were obtained indicating the lack of the matrix effect on quantification of the analytes. This assessment is necessary when analogues rather than a stable isotope labeled parent compounds are utilized as internal standards.

Following the procedure described in Section 2, the assay was validated in human plasma in the concentration range of 0.1 to 100 ng/ml. For nine

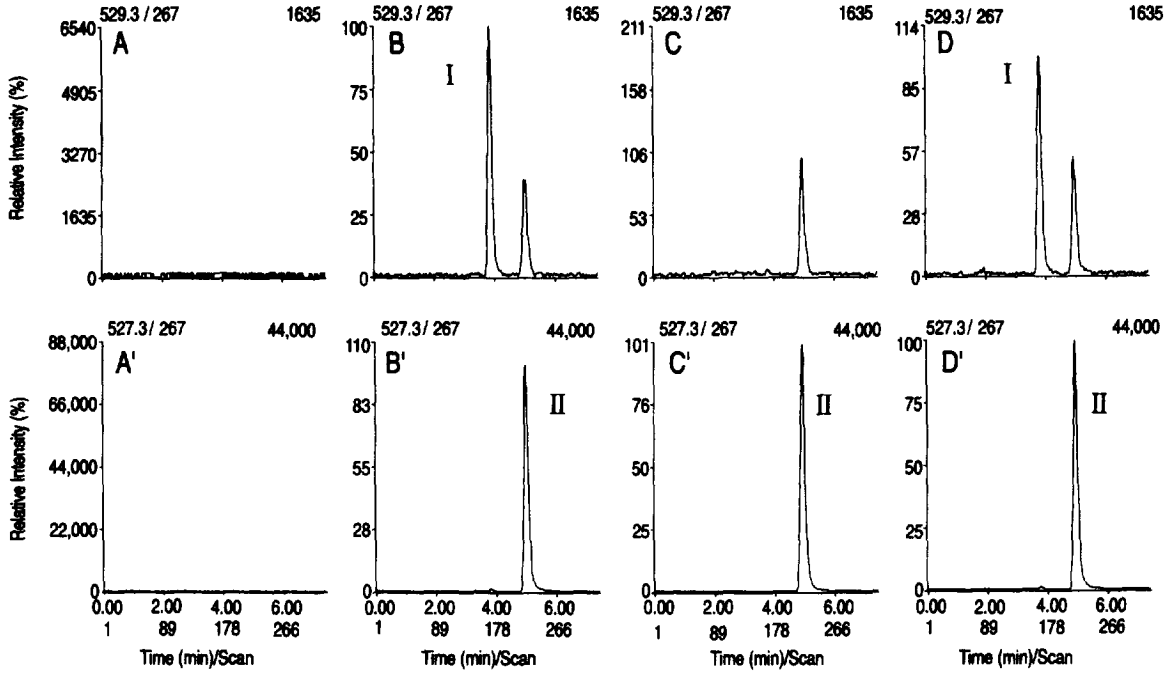

Fig. 3. Representative HPLC–MS–MS chromatograms of plasma (1 ml) extracts obtained by multiple reaction monitoring at m/z 529→267 (channel "a") for I and m/z 527→267 (channel "b") for II; (A, A') blank control plasma monitored at channels "a" and "b", respectively; (B, B') control plasma spiked with 0.5 ng of I and 10 ng of II monitored at channels "a" and "b", respectively; (C, C') predose plasma sample of a subject spiked with 10 ng of II monitored at channels "a" and "b"; (D, D') plasma sample of a subject 16 h after receiving a 5-mg oral dose of I spiked with 10 ng II and monitored at channels "a" and "b"; concentration of I equivalent to 0.4 ng/ml. The numbers in upper right hand corner of chromatograms correspond to the peak heights expressed in arbitrary units. There was a small "a cross-talk" effect observed between the channels used for quantification of I and II, and each compound gave small response in the channel used for quantification of the other compound. Specificity of the method was assured by providing chromatographic separation of I from II.

daily runs, the mean slope and correlation coefficient of the calibration lines were 0.696±0.00449 and 0.996±0.002, respectively. The difference between the nominal standard concentration and the back-calculated concentration from the weighted linear regression line was less than 10% for each point on the standard curve indicating that the linear regression applied ($1/y^2$) provided an acceptable fit of the data. Correlation coefficient was always better than 0.99. Representative chromatograms are shown in Fig. 3.

The intra-day precision, expressed as the coefficient of variation (C.V., %), was less than 7% at all concentrations within the standard curve range (Table 1).

The LOQ of the assay in plasma was 0.1 ng/ml. The LOQ was defined here as the lowest concentration on the standard curve for which the precision of determination, expressed as coefficient of variation (C.V., %), was less than 10%, with an adequate assay accuracy (100±10%). In addition, the analyses of QC standards indicated that I was stable in plasma for at least four months when stored at −20°C (Table 2).

The performance of the HPLC–MS–MS method was tested by analyzing more than 3000 plasma samples from a number of clinical studies with I. As an example, representative concentrations of I in plasma of selected human subjects participating in a single dose pharmacokinetic study, after oral administration of I, are presented in Table 3.

In conclusion, the HPLC–MS–MS method for the determination of I in human plasma with the LOQ of 0.1 ng/ml was developed and its ruggedness and long-term performance was confirmed during analyses of I in plasma samples originating from a number of clinical trails. In comparison with a conventional method based on HPLC with fluorescence detection, the HPLC–MS–MS method offers better sensitivity, allows a significant simplification and shortening of the sample isolation procedure and chromatography and considerably improves sample throughput and overall speed of analysis.

Table 1
Precision and accuracy data for the determination of I in human plasma

Nominal concentration (ng/ml)	Mean[a] concentration (ng/ml)	Precision,[b] C.V. (%)	Accuracy[c] (%)
0.10	0.10	4.0	100
0.20	0.24	2.9	96
0.50	0.49	5.0	98
1.00	1.01	4.1	101
2.50	2.71	3.7	108
5.00	5.16	3.5	103
10.00	10.10	6.1	101
50.00	49.68	4.8	99
100.00	95.21	1.7	95

[a] Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.
[b] Expressed as coefficient of variation (C.V.%, $n=5$).
[c] Expressed as [(mean observed concentrations)/(nominal concentration)×100], ($n=5$).

Table 2
Inter-day variability for the assay of quality control samples spiked with I

Initial[a] concentration (ng/ml)	n	Mean calculated concentration (ng/ml)	C.V. (%)
0.89	18[b]	0.92	8.7
73.3	18[b]	73.5	7.6

[a] $n=5$.
[b] Over a period of 9 days. Both low and high QC samples were analyzed after four months of storage at −20°C. Mean values ($n=5$) obtained were (0.94±0.02) and (73.1±0.8) ng/ml indicating the adequate stability of I during storage at −20°C.

Table 3
Concentration of I in plasma (ng/ml) of selected human subjects after oral administration of 5 mg of I

Time post-dose (h)	Concentration of I (ng/ml)	
	Subject 2	Subject 5
0	0	0
0.5	2.04	0.65
1.0	8.02	2.24
1.5	6.76	2.03
2.0	4.80	1.82
4.0	2.58	0.94
8.0	1.61	0.63
12.0	0.92	0.48
24.0	0.15	0.11

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