ELSEVIER

Journal of Chromatography B, 693 (1997) 117–129

JOURNAL OF CHROMATOGRAPHY B

Low level determination of a novel 4-azasteroid and its carboxylic acid metabolite in human plasma and semen using high-performance liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry

M.L. Constanzer[a], C.M. Chavez[a], B.K. Matuszewski[a,*], J. Carlin[b], D. Graham[b]

[a]*Merck Research Laboratories, West Point, PA 19486, USA*
[b]*Merck Research Laboratories, Rahway, NJ 07065-0900, USA*

Received 9 September 1996; revised 2 December 1996; accepted 9 December 1996

Abstract

Compound **I** (4,7β-dimethyl-4-azacholestan-3-one, MK-0386) is a potent 5α-reductase type 1 (5αR1) inhibitor. Sensitive (0.2 ng/ml), specific and separate assays have been developed and validated for the analysis of **I** and its carboxylic acid metabolite (**II**) in human semen and plasma based on high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS–MS) detection. After liquid–liquid extraction of the analytes from biological matrix, the extracts were chromatographed on a short (50 mm) analytical column during analysis of **I**, and on a longer (150 mm) column with a weaker mobile phase during the analysis of **II**. This additional chromatographic separation was required to separate **II** from a secondary metabolite present in post-dose plasma samples interfering with the quantification of **II**. The MS–MS detection was performed on a Sciex API III Plus tandem mass spectrometer using the heated nebulizer probe. Monitoring the parent→product ion combinations of m/z 416→114 and 404→114, in the multiple reaction monitoring (MRM) mode, after chromatographic separation, allowed quantification of both analytes. The standard curve in plasma was linear in the concentration range of 0.2 to 200 ng/ml for both **I** and **II**, with correlation coefficients greater than 0.99 and coefficients of variation of less than 15% for replicate ($n=5$) analysis at all concentrations within the standard curve range. For the semen assay the linear range for determination of **I** was from 0.2 to 50 ng/ml. These assays were applied to support a number of clinical studies with **I** and their validity and long-term performance was confirmed during analyses of clinical samples from these studies. The need for careful assessment of the specificity of MS–MS assays in post-dose biological fluid samples in the presence of metabolites was emphasized.

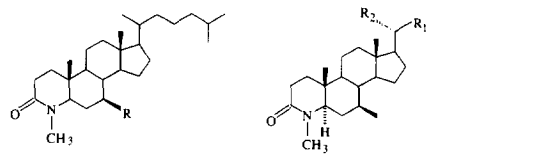
Keywords: Steroids; 4-Azasteroid

1. Introduction

Compound **I** (MK-0386, Fig. 1) belong to a class of 4-azasteroids with a chemical structure similar to finasteride, a potent 5α-reductase (5αR) inhibitor [1]. Inhibition of 5αR results in an antiandrogen effect by decreasing target organ dihydrotestosterone (DHT) levels. Two isozymes of 5αR are known to exist, type 1 (5αR1) and type 2 (5αR2). In contrast to finasteride, which is a potent inhibitor of 5αR2,

*Corresponding author.

PII S0378-4347(97)00047-9



I: R = CH_3
III: R = H

II: R_1 = CH_2CH_2COOH; R_2 = CH_3
IV: R_1 = COOH; R_2 = H

Fig. 1. Chemical structures of **I**, **II** and internal standards **III** and **IV**.

compound **I** is a potent and specific inhibitor of the human 5αR1 [2,3]. Finasteride has undergone successful clinical evaluation [4], and was approved for use in the treatment of benign prostatic hyperplasia (BPH). In a multiple oral study for 7 days, finasteride at 1 and 10 mg doses reduced the serum DHT levels 71% and 73%, respectively [5], and much of the residual DHT could be due to production by the type 1 enzyme which was not effectively inhibited by finasteride, an effective inhibitor of 5αR2. Inhibitors of 5αR1 in combination with inhibitors of 5αR2 could lower residual DHT levels further and thus might afford a more effective clinical treatment of BPH. The validity of this hypothesis was confirmed in a clinical study in which finasteride (5 mg) and **I** (25 mg) were given orally in combination, leading to nearly complete suppression of circulating DHT concentrations [6]. The localization of the 5αR1 in the sebaceous gland of skin [7] and the implication that DHT is the perquisites for the onset of acne suggest that inhibition of this isoenzyme may be useful in the management of this disorder.

In order to support clinical pharmacokinetic studies with the 5αR1 inhibitor **I**, it was necessary to develop sensitive and specific methods for the determination of **I** and its carboxylic acid metabolite **II** in human plasma and semen with the limit of quantification (LOQ) of less than 1 ng/ml. Due to the lack of good chromophore and poor absorbance of **I** and **II** in the ultraviolet (UV) region of the spectrum, the development of an assay based on HPLC with UV absorption (210 nm) detection at sub-nanogram levels was not feasible, as demonstrated earlier during the development of an assay for finasteride in human plasma [8]. Based on our previous experience with this compound [9], the HPLC–MS–MS method was evaluated for the feasibility of quantification of **I** and **II** at LOQ<1 ng/ml. In addition to the successful development of an assay for finasteride in human plasma with the LOQ of 0.2 ng/ml [9], the HPLC–MS–MS methodology was also utilized in preclinical studies for determination of other azasteroids in plasma at concentrations down to 0.5 ng/ml [10,11].

The subject of this paper is the development of highly sensitive HPLC–MS–MS assays for the determination of **I** and **II** in human plasma and semen with the LOQ of 0.2 ng/ml for both analytes. The need for careful assessment of the specificity of MS–MS based assays in the presence of metabolites is also emphasized.

2. Experimental

2.1. Materials

Compounds **I**, **II**, **III** and **IV** were synthesized at 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 and semen originated from Biological Specialties Corporation (Lansdale, PA, USA). Air (hydrocarbon-free), nitrogen (99.999%) and argon (99.999%) was purchased from West Point Supply (West Point, PA, USA).

2.2. Instrumentation

A PE-Sciex (Thornhill, Ontario, Canada) API III^+ tandem mass spectrometer equipped with a heated nebulizer interface, a Waters (Waters-Millipore, Milford, MA, USA) WISP 715 autoinjector and a Perkin-Elmer biocompatiable binary pump (Model 250) were used for all HPLC–MS–MS analyses. The data was processed using MacQuan software (PE-Sciex) on a MacIntosh Quadra 900 microcomputer. HPLC analyses were performed on an Inertsil C-8 50×4.6 mm I.D., 5-μm analytical column (Meta-Chem Technologies, Torrance, CA, USA) coupled with a 2-μm in-line filter heated to 60°C (column 1), or on a C_8 BDS 150×4.6 mm I.D. 5-μm analytical column (column 2, Keystone Scientific, Bellefonte, PA, USA).

2.3. Standard solutions

A stock solution (1 mg/ml) of **I** and **II** was prepared independently in methanol. These solutions were further diluted with methanol to give a series of working standards with the concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 100.0 ng/ml for plasma assay. The internal standards **III** and **IV** were also prepared as a stock solution (1 mg/ml) in methanol by dissolving 10 mg of solid **III** or **IV** in 10 ml of methanol. A working standard of 10.0 ng/ml was prepared by serial dilutions of stock standards with methanol, and were used for all analyses. All standards were prepared once a month and stored at 5°C.

A series of quality control (QC) samples at 1 and 150 ng/ml for **I** and 2 and 150 ng/ml for **II** were prepared in plasma. 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 in order to assess inter-day assay performance.

2.4. Chromatographic conditions

Three separate methods, A, B and C, were evaluated and utilized for determination of **I** and **II**. Method A was used originally for simultaneous determination of **I** and **II** in control plasma, whereas methods B and C were used later for separate determination of **I** and **II**, respectively. Chromatographic conditions were the same in methods A and B, but different in method C. In method A and B the mobile phase was a mixture of 90% acetonitrile and 10% water containing 0.1% formic acid and 10 m*M* ammonium acetate, and was delivered to column 1 at a flow-rate of 1 ml/min. The retention times for **I** and **II** were 2.7 and 0.8 min. In method C the mobile phase was acetonitrile–water (75:25, v/v) containing 10 m*M* ammonium acetate and 0.1% formic acid, respectively. The flow-rate in method C was 1.5 ml/min and the analytical column 2 was utilized. The retention time of **II** and **IV** in method C was 5.5 and 2.5 min, respectively. The aqueous portion of the mobile phase was prepared by dissolving 0.77 g of ammonium acetate in 1000 ml of water and the addition of 820 μl formic acid.

In addition, the separation of metabolites interfering with quantification of **II**, and a mass spectrum (MS) and product (MS–MS) ion mass spectra of the interfering metabolite were obtained under gradient chromatographic conditions using column **2** (solvent "A": acetonitrile–0.1% formic acid and 10 m*M* ammonium acetate in water (90:10, v/v), solvent "B," the same solution as "A" but at 50:50, v/v, mobile phase gradient: 100% "B" for 9 min at 1.0 ml/min, 100% "A" for 0.5 min at 1.0 ml/min and 100% "A" for 10 min at 1.5 ml/min).

2.5. Sample preparation

Sample preparation was based on a liquid–liquid extraction and was different in methods A, B and C.

In method A, a 1-ml aliquot of plasma was pipetted into a 15-ml centrifuge tube and 100 μl of working standard solutions of **III** and **IV** (equivalent to 10 ng/ml of each internal standard) were added, followed by the addition of 1 ml of 0.2 *M* citrate buffer (pH 2.8) and 7 ml of methyl-*tert.*-butyl ether (MTBE). 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 a clean centrifuge tube. The organic extract was evaporated to dryness under a stream of nitrogen at 50°C, the residue was reconstituted in 300 μl mixture consisting of acetonitrile–water (85:15, v/v) containing 0.1% formic acid and 10 m*M* ammonium acetate, and after vortexing for 1 min and sonicating for 15 min, a 100-μl aliquot was injected into the HPLC–MS–MS system.

In method B, 1 ml of plasma or semen was deproteinized with 2 ml of acetonitrile and then extracted with 5 ml of hexane. After rotate-mixing for 15 min, the organic layer was transferred to a clean centrifuge tube and evaporated to dryness. The residue was reconstituted in 300 μl of the same mixture as in method A, and a 150-μl aliquot was injected into the HPLC–MS–MS system.

In method C, the extraction procedure was the same as in method A, but only internal standard **IV** was used. The residue after liquid–liquid extraction and evaporation of the organic layer to dryness was reconstituted in 300 μl mixture consisting of acetonitrile–water (65:35, v/v) containing 0.1% formic acid

10 m*M* ammonium acetate, and a 100-μl aliquot was injected for analysis.

2.6. HPLC–MS–MS conditions

A PE-Sciex triple quadrupole mass spectrometer was interfaced via a Sciex heated nebulizer probe to HPLC system, and atmospheric pressure chemical ionization was affected by a corona discharge needle (+4 μA) using positive ion atmospheric pressure chemical ionization. The heated nebulizer probe was maintained at 500°C. The nebulizing gas (air) pressure and auxiliary flows were set at 80 p.s.i. and 2 l/min, respectively (1 p.s.i.=6894.76 Pa). Curtain gas flow (nitrogen) was 0.9 l/min, and the sampling orifice potential was set at +60 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 molecules $[M+H]^+$ at *m/z* 416 for **I**, *m/z* 404 for **II**, *m/z* 402 for **III** and *m/z* 362 for **IV**, via the first quadrupole filter (Q1), with collision-induced fragmentation at Q2 (collision gas argon, $320 \cdot 10^{13}$ atoms cm^{-2}), and monitoring the product ions via Q3 at *m/z* 114 for all analytes. The electron multiplier setting was −4.2 kV. Peak-area ratios [(*m/z* 416→114)/(*m/z* 402→114)] for **I** and [(*m/z* 404→114)/(*m/z* 362 →114)] for **II**, obtained from multiple reaction monitoring of analytes were utilized for the construction of calibration curves, using weighted $(1/y^2)$ linear least square regression of the plasma and semen concentrations and the measured area ratios. Data collection, peak integration and calculations were performed using MacQuan PE-Sciex software.

2.7. Precision, accuracy, recovery and specificity

The precision of the method was determined by the replicate analyses ($n=5$) of human plasma or semen containing **I** or **II** 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 the 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** or **II** extracted from biological fluids to that of standards injected directly.

3. Results and discussion

A number of different analytical approaches for the determination of **I** and **II** were initially evaluated in our and other groups [12,13] and included HPLC with UV absorption detection, capillary gas chromatography with negative ion chemical ionization mass spectrometric (GC–NICI-MS) detection [12] and HPLC with MS–MS detection [13]. The HPLC–UV method was not sensitive enough for low level determination of **I** and **II**, and the HPLC–MS–MS assays, initially developed for preclinical evaluation of **I** in rat and rhesus monkey plasma, had a LOQ of ≥1 ng/ml [13]. The GC–NICI-MS approach seemed to provide sensitivities below 1 ng/ml for both analytes [12] but required double derivatization with heptafluorobutyric anhydride and diazomethane. Reproducibility of these derivatization reactions on a day-to-day basis and the precision and accuracy of the method were not fully assessed. Therefore, in order to support human pharmacokinetic studies with **I** our efforts concentrated on the development of HPLC–MS–MS methods in human plasma in semen with the required LOQ of 0.2 ng/ml. In addition to a significant improvement in assay sensitivity, the methods described here were subjected to an extensive and stringent validation procedures and their specificity in post-dose human plasma samples in the presence of metabolites was assessed. These studies indicated the presence of partially coeluting metabolite(s) interfering with the quantification of **II**, and this finding led to the important modifications to our original assay procedure for **II**.

The positive product mass spectra of the protonated molecules $[(M+H)^+]$ of **I** (*m/z* 416), **II** (*m/z* 404), **III** (*m/z* 402) and **IV** (*m/z* 362) indicated the presence of a common product ion at *m/z* 114 for all analytes (Fig. 2).

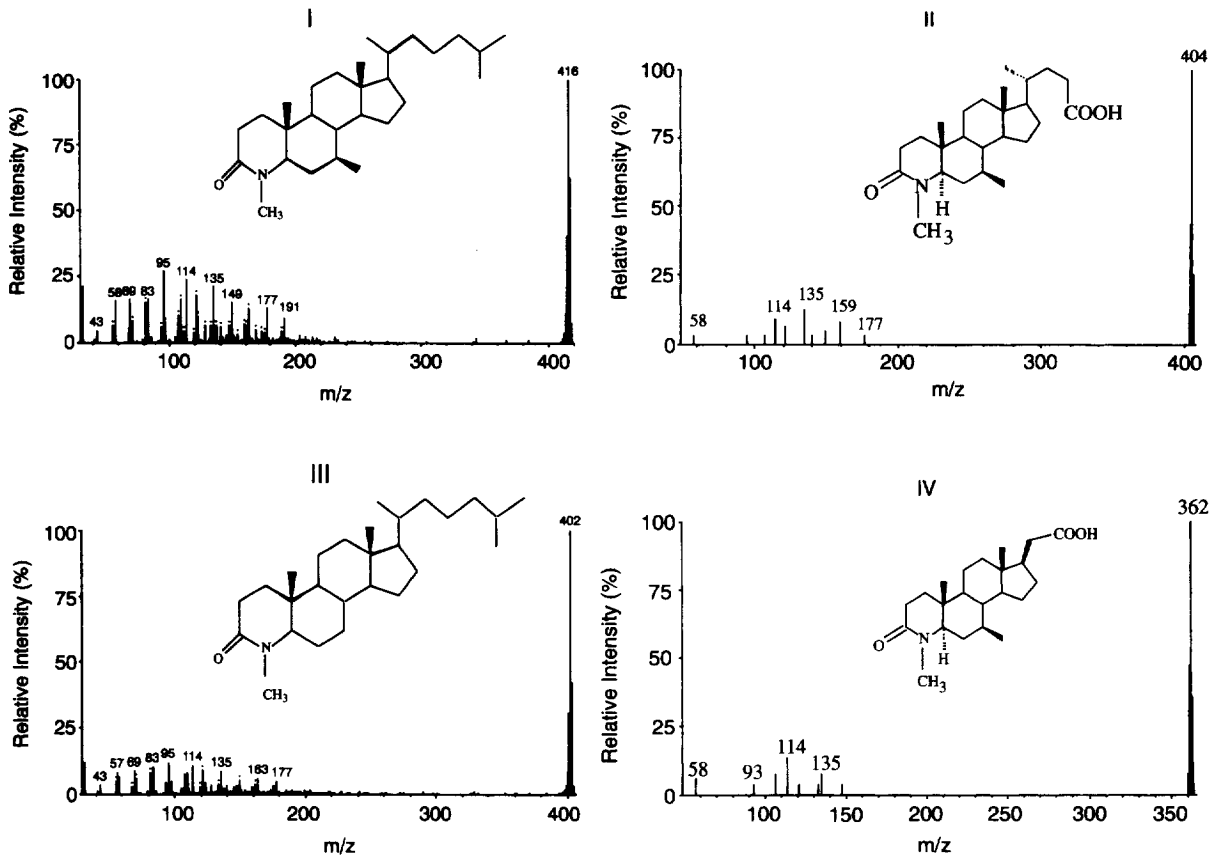


Fig. 2. Positive product ion mass spectra of the protonated molecules of **I** (*m/z* 416, A), **II** (*m/z* 404, B) and internal standards **III** (*m/z* 402, C) and **IV** (*m/z* 362, D).

By monitoring the parent→product ion pairs at *m/z* 416→114 for **I**, *m/z* 404→114 for **II**, *m/z* 402→114 for **III** and *m/z* 362→114 for **IV** in the multiple reaction monitoring mode, highly sensitive assays for **I** and **II** in plasma and **I** in semen with the LOQ of 0.2 ng/ml were developed. The product ion at *m/z* 114 was chosen for quantification based on the absence of interferences in extracts of plasma or semen samples originating from different sources.

3.1. Simultaneous determination of I and II in control plasma (method A)

Initially, an attempt was made to develop a single assay for the simultaneous determination of both **I** and **II**. Due to the significant difference in polarity between **I** and **II**, the retention time for the carboxylic acid metabolite **II** was much shorter than the retention time of a more hydrophobic **I**. In order to perform multi-sample analyses with the desired short analysis time (≤5 min), chromatography was performed on a short (50 mm) column 1 (Section 2.2). The retention times for **II** and **I** were 0.8 and 2.7 min, respectively, and the metabolite **II** was poorly retained on the column (capacity factor $k'<1$). Following the procedure described in Section 2.4, the assay for the simultaneous determination of **I** and **II** was validated in control plasma in the concentration range of 0.25 to 100 ng/ml for both analytes. The run time of the method was 3.5 min. The intra-day precision, expressed as the coefficient of variation (C.V., %), was assessed in five different lots of plasma and was less than 10.6% at all concentrations within the standard curve range (Table 1). The

Table 1
Intra-day variability of the simultaneous determination of **I** and **II** in control plasma (method A); precision and accuracy of replicate analysis ($n=5$)

Nominal concentration (ng/ml)	**I**			**II**		
	Precision[a] (C.V., %)	Mean[b] concentration (ng/ml)	Accuracy[c] (%)	Precision[a] (C.V., %)	Mean[b] concentration (ng/ml)	Accuracy[c] (%)
0.25	5.3	0.25	100	10.6	0.26	104
0.50	7.3	0.53	106	6.5	0.49	98
1.00	3.8	0.99	99	9.3	0.97	97
2.50	7.1	2.49	100	6.4	2.38	95
5.00	4.2	4.89	98	2.3	4.82	96
10.00	3.2	10.13	101	3.0	9.76	98
25.00	3.6	24.45	102	2.4	26.77	107
50.00	3.6	48.62	97	3.1	51.46	103
100.00	6.0	103.16	103	2.8	108.7	109

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

accuracy of the method varied from 97 to 106% for **I**, and 95 to 109% for **II**. Representative chromatograms are presented in Figs. 3 and 4. The absence of any "cross talk" between the channels used for quantification of **I**, **II**, **III** and **IV** was demonstrated by injecting 100 ng/ml samples of **I** and/or **II** and

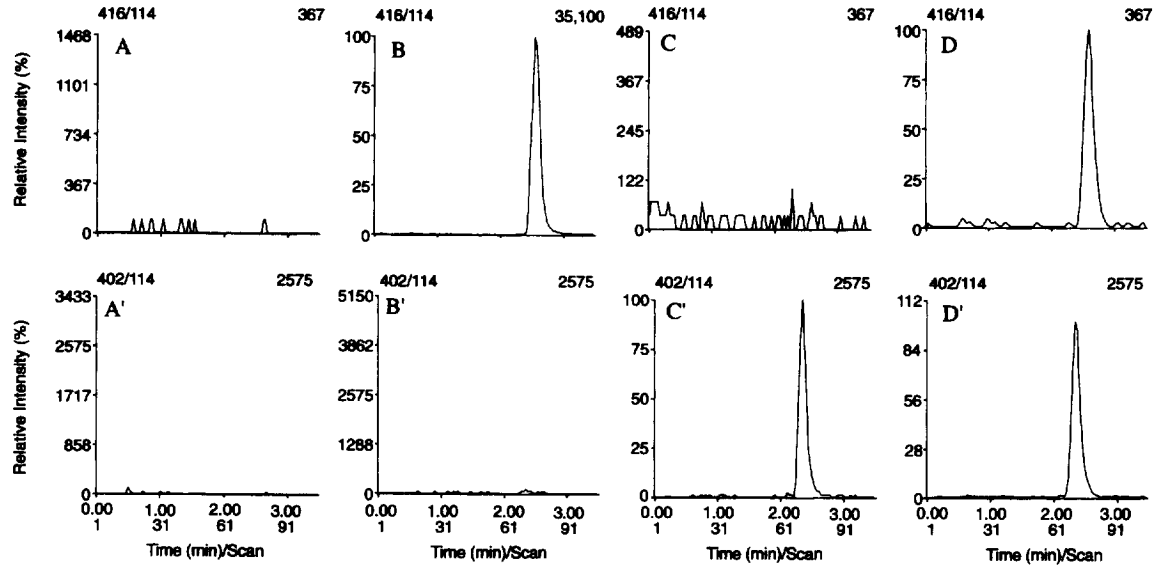


Fig. 3. Representative HPLC–MS–MS chromatograms of plasma (1 ml) extracts obtained by multiple reaction monitoring at m/z 416→114 (channel "a") for **I** and m/z 402→114 (channel "b") for internal standard **III**; using method A: (A, A') blank control plasma monitored at channels "a" and "b", respectively; (B, B') control plasma spiked with 100 ng of **I** monitored at channels "a" and "b", respectively; (C, C') control plasma spiked with 10 ng of **III** monitored at channels "a" and "b", respectively; (D, D') control plasma spiked with 1 ng of **I** and 10 ng of **II** monitored at channels "a" and "b", respectively. The numbers in upper right hand corner correspond to the peak heights expressed in arbitrary units.

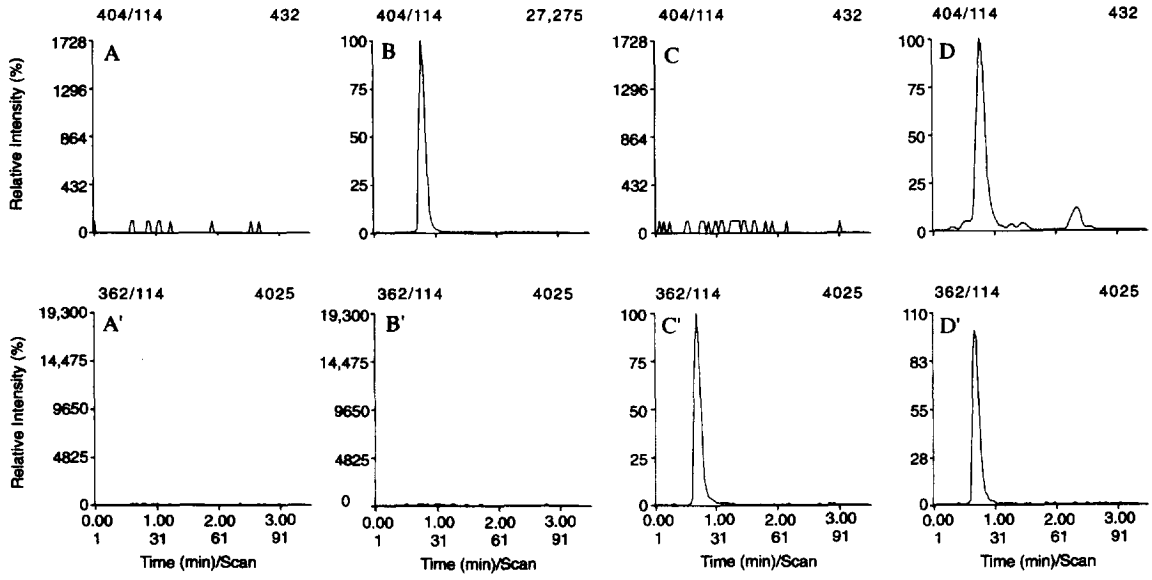


Fig. 4. Representative HPLC–MS–MS chromatograms of plasma (1 ml) extracts obtained by multiple reaction monitoring at m/z 404→114 (channel "a") for **II** and m/z 362→114 (channel "b") for internal standard **IV**; using method A: (A, A′) blank control plasma monitored at channels "a" and "b", respectively; (B, B′) control plasma spiked with 100 ng of **II** monitored at channels "a" and "b", respectively; (C, C′) control plasma spiked with 10 ng of **IV** monitored at channels "a" and "b", respectively; (D, D′) control plasma spiked with 2.5 ng of **II** and 10 ng of **IV** monitored at channels "a" and "b", respectively. The numbers in upper right hand corner correspond to peak heights expressed in arbitrary units.

monitoring the response at channels used for determination of **III** and **IV** (Figs. 3B, 3B′, 4B and 4B′). In addition, internal standards **III** and **IV** spiked at 10 ng/ml (Figs. 3C′ and 4C′) gave no response at channels used for quantification of **I** and **II** (Figs. 3C and 4C).

3.2. Analysis of plasma samples of subjects dosed with I using method A

Selected post-dose plasma samples from subjects receiving 50-‘ and 100-mg single oral doses of **I** were analyzed for the presence of **I** and **II** using method A, but an interfering peak M partially coeluting with **II**, and giving response at the same m/z 404→114 channel as used for quantification of **II**, was observed.

This indicated that the combination assay validated in control plasma was not specific for the quantification of **II** in post-dose plasma samples and required separation of **II** from a metabolite(s) M coeluting with the peak of interest. Although the retention of **II** on column 1 was very poor ($k'<1$), even this limited retention allowed partial separation of **II** from an interfering metabolite peak (Fig. 5). If no retention of **II** was provided, the presence of an interference from a metabolite(s) would go undetected.

In order to obtain some information about the nature of the interfering metabolite(s) present in post-dose plasma potentially interfering with the quantification of **II**, selected pre- and post-dose samples were analyzed under gradient chromatographic conditions (Section 2.4). The mass spectrum (MS) of plasma extracts were obtained and selected ion chromatograms were extracted from these spectra. These extracted ion chromatograms indicated that the interfering peak M, eluting at the retention time (t_R) of 3.3 min and fully separated here from **II** (t_R 7.4 min), corresponded to a compound with the $(M+H)^+$ at m/z 461 (Fig. 6). This compound gave a fragment at m/z 404 at the same t_R (3.3 min) as the interfering metabolite (Figs. 6 and 7A). The retention time for **I** under these conditions was 17.0 min (m/z 416).

The mass spectrum (Fig. 7A) and product mass

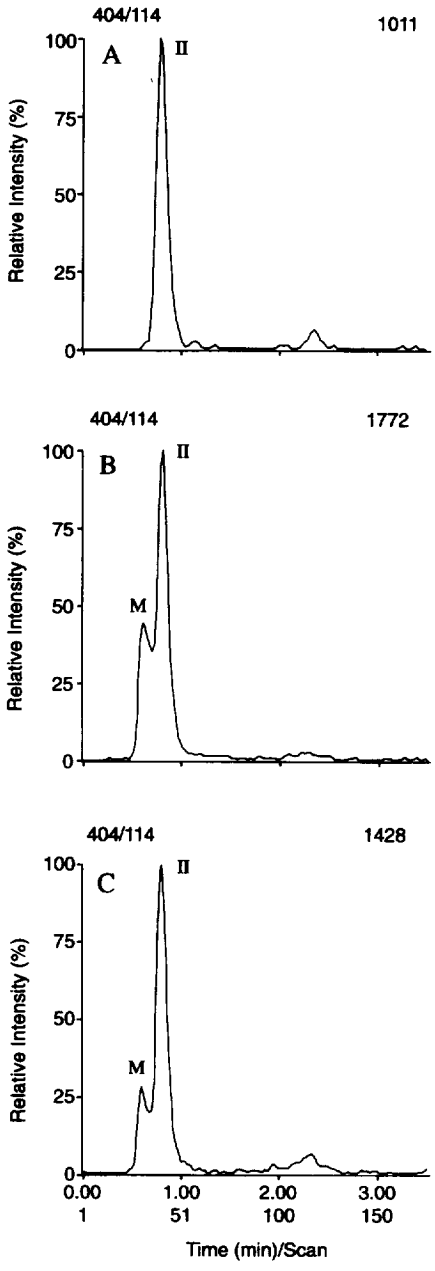


Fig. 5. Representative HPLC–MS–MS chromatograms of plasma (1 ml) extracts obtained by monitoring at m/z 404→114 using method A; (A) control plasma spiked with 5 ng/ml of **II**; (B) post-dose plasma sample of a subject 36 h after oral administration of 50 mg of **I**; (C) as B, 48 h after oral administration of 50 mg of **I**.

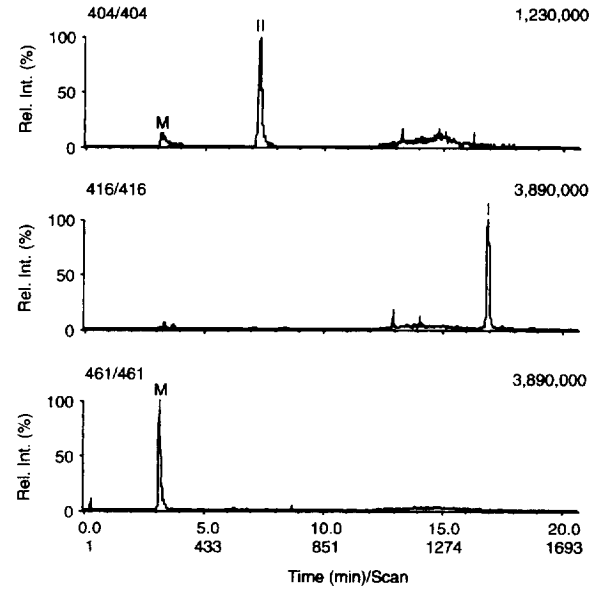


Fig. 6. Extracted mass chromatograms (Q1 scan) at m/z 461, 404 (**II**) and 416 (**I**) under gradient chromatographic conditions (Section 2.4).

spectra (Fig. 7B) of the protonated molecule of M at m/z 461 are shown in Fig. 7. The mass spectrum (Fig. 7A) confirmed that a fragment ion of a very low intensity at m/z 404, originating from the $(M+H)^+$ ion of M, was present. The fragmentation patterns observed for M at m/z 461 (Fig. 7B) and for **II** at m/z 404 (Fig. 2) were very similar except for the presence of a fragment at m/z 76 in M which was absent in a spectrum of **II**. The structure of M cannot be unequivocally ascertained based on these data alone, but one can postulate that M corresponded to a glycine conjugate of **II** similar to the glycocholic acid conjugate formed during the metabolism of cholesterol. This hypothesis is based on the presence of $(M+H)^+$ at m/z 461 and a fragment ion at m/z 76 in the product ion spectrum of M which is probably formed from $(M+H)^+$ through α-cleavage and the elimination of a ketene (Fig. 7B), an important decomposition pathway in multi functional acetamides.

Therefore, the combination assay for the determination of **I** and **II** validated in control plasma using method **A** had to be modified to separate chromatographically the metabolite M from **II**.

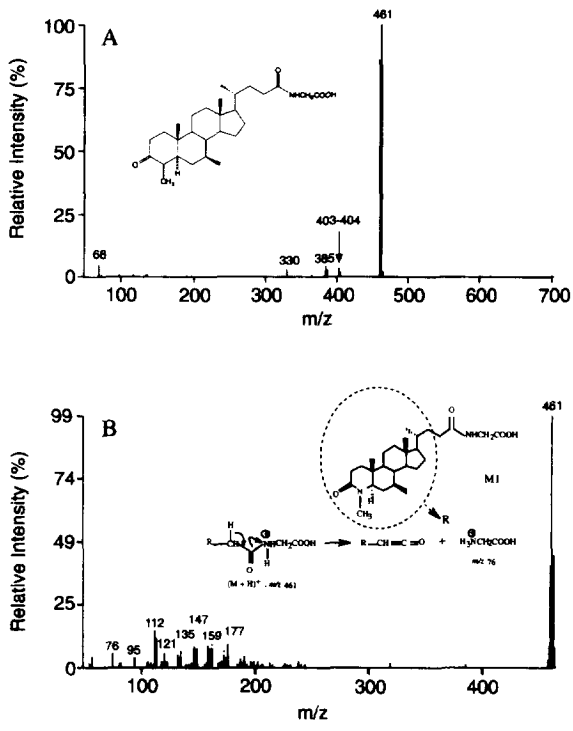


Fig. 7. The mass spectrum (A) and product mass spectrum (B) of $(M+H)^+$ ion at m/z 461 of metabolite **M** present in pooled post-dose plasma sample of several subjects dosed with 100 mg of **I**. The analogous product mass spectrum of the $(M+H)^+$ ion of **II** at m/z 404 is shown in Fig. 2.

3.3. Separate methods for determination of I (method B) and II (method C)

Method B: The extraction procedure from plasma and/or semen for separate determination of **I** was simplified in comparison with method A. Liquid–liquid extraction with hexane after protein precipitation with acetonitrile was utilized, and the adjustment of pH of plasma to 2.8, necessary for efficient extraction of **II**, was not required. Using the same chromatographic conditions as in method A, the assay for **I** was validated in the concentration range of 0.2 to 200 ng/ml in plasma and 0.2 to 50 ng/ml in semen. The intra-day precision and accuracy data are presented in Tables 2 and 3, respectively.

The limit of quantification (LOQ) in both plasma and semen was 0.2 ng/ml and was defined as the lowest concentration on the standard curve for which precision of the determination, expressed as coefficient of variation (C.V., %), was less than 15%, with an adequate assay accuracy (100±15%). The inter-day day precision, as measured by the concentration of QC standards, was below 7% (Table 4).

Typical equation for the calibration curves for **I** in plasma was $y=0.147371x-0.00133$, with the correlation coefficients of >0.99. Representative chromatograms are shown in Fig. 8.

Table 2
Intra-day variability of the separate determination of **I** and **II** in human plasma using methods B and C, respectively; precision and accuracy of replicate analysis ($n=5$)

Nominal concentration (ng/ml)	**I**			**II**		
	Mean[a] concentration (ng/ml)	Precision[b] (C.V., %)	Accuracy[c] (%)	Mean[a] concentration (ng/ml)	Precision[b] (C.V., %)	Accuracy[c] (%)
0.20	0.20	3.2	100	0.21	13.6	105
0.50	0.49	6.7	98	0.46	2.7	92
1.00	0.99	5.8	99	0.93	5.9	93
2.50	2.46	1.6	98	2.43	5.2	97
5.00	4.94	2.9	99	5.28	8.6	106
10.00	9.97	6.1	100	10.67	5.1	107
25.00	24.60	4.3	98	24.56	5.1	98
50.00	50.05	3.9	100	53.56	2.0	107
100.00	104.70	6.5	105	100.99	5.5	101
200.00	209.24	6.9	105	210.64	7.4	105

[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.%).

[c] Expressed as [(mean observed concentrations)/(nominal concentration)×100], ($n=5$).

Table 3
Intra-day variability of the determination of **I** in semen using method B; precision and accuracy of replicate analysis ($n=5$)

Nominal concentration (ng/ml)	Mean[a] concentration (ng/ml)	Precision[b] (C.V., %)	Accuracy[c] (%)
0.20	0.21	9.3	105
0.50	0.49	7.4	98
1.00	1.00	3.0	100
5.00	5.05	1.0	101
10.00	10.25	1.7	102
25.00	24.87	3.0	100
50.00	49.67	2.2	99

[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.%).
[c] Expressed as [(mean observed concentrations)/(nominal concentration)×100], ($n=5$).

Method C: The extraction procedure was the same as in method A, but the residue after evaporation of the extraction solvent to dryness was reconstituted in a weaker mobile phase (see Section 2.5) and injected on longer column 2 using a mobile phase with a much lower content of the organic solvent. Under the chromatographic conditions utilized in method C, analyte **II** had the t_R of 5.5 min and was well retained ($k' \approx 4.7$) and well separated from M. The total analysis time was 6.5 min. Typical equation for the calibration curve for **II** in plasma was $y = 0.066971x - 0.007334$ with the correlation coefficients of >0.99. The intra- and inter-day precision and accuracy data are presented in Tables 2 and 4, and representative chromatograms are shown in Fig. 9.

3.4. Analyses of samples from clinical studies

Methods B and C were used to support clinical pharmacokinetic studies with **I**. As an example, representative concentrations of **I** and **II** in plasma of selected human subjects after single oral administration of 25 mg of **I** are presented in Table 5.

3.5. Comments about specificity of HPLC–MS–MS assays in biological fluids

It is generally assumed that due to the high specificity of tandem mass spectrometric detection the specificity of HPLC–MS–MS assays is virtually assured, and none or little chromatographic separation is necessary, The assessment of assay specificity is usually based only on the analysis of a drug and an internal standard in a control or pre-dose biological fluid samples. However, contrary to this common believe, we have demonstrated [14] that careful assessment of assay specificity in post-dose biological fluid samples in the presence of metabolites is necessary. The commonly formed metabolite(s) coeluting with parent compound has the potential of fragmenting to some extent in the APCI region of the MS system giving fragment ions in mass spectra which may be characterized by the same m/z ratio as the protonated molecular ions of either parent compound or an internal standard. Further fragmentation in Q2 of these ions originating from metabolites leads to the formation of product ions which are the same as product ions of a parent compound or an internal standard utilized for quantification. The net result is the potential interference of a metabolite(s) with the quantification of a parent

Table 4
Inter-day variability for the separate assays of quality control samples spiked with **I** (method B) and **II** (method C)

Analyte	Initial[a] concentration (ng/ml)	Number of determinations	Mean calculated concentrations (ng/ml)	C.V. (%)
I	1.0	4[b]	1.0	3.7
I	151.8	4[b]	142.4	6.3
II	1.76	6[c]	1.86	6.1
II	155.5	6[c]	165.3	6.5

[a] $n=5$.
[b] Over a period of 2 days.
[c] Over a period of 3 days.

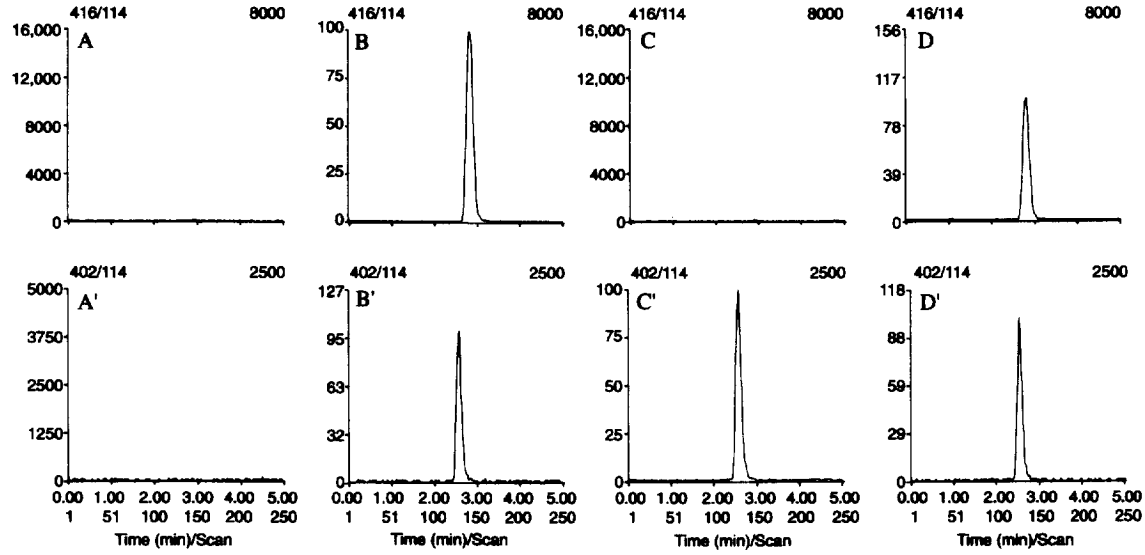


Fig. 8. Representative HPLC–MS–MS chromatograms of plasma (1 ml) extracts obtained by multiple reaction monitoring at m/z 416→114 (channel "a") for **I** and m/z 402→114 (channel "b") for internal standard **III** using method B; (A, A′) blank control plasma monitored at channels "a" and "b", respectively; (B, B′) control plasma spiked with 50 ng of **I** and 50 ng of **III** monitored at channels "a" and "b", respectively; (C, C′) pre-dose plasma sample of a subject spiked with 50 ng of **III** monitored at channels "a" and "b", respectively; (D, D′) post-dose plasma sample of a subject (day 14, 8 h post-dose) spiked with 50 ng of **III**, concentration of **I** equivalent to 31 ng/ml. The numbers in upper right hand corner correspond to the peak heights expressed in arbitrary units.

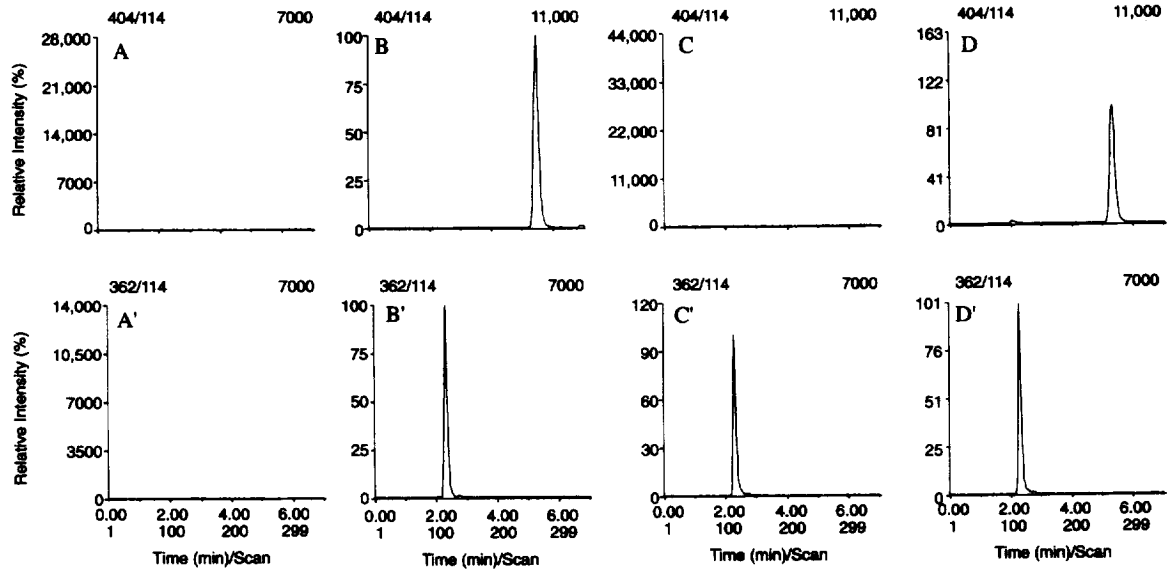


Fig. 9. Representative HPLC–MS–MS chromatograms of plasma (1 ml) extracts obtained by multiple reaction monitoring at m/z 404→114 (channel "a") for **II** and m/z 362→114 (channel "b") for **IV** using method C; (A, A′) blank control plasma monitored at channels "a" and "b", respectively; (B, B′) control plasma spiked with 50 ng of **II** and 50 ng of **IV** monitored at channels "a" and "b", respectively; (C, C′) pre-dose plasma sample of a subject spiked with 50 ng of **IV** monitored at channels "a" and "b", respectively; (D, D′) post-dose plasma sample of a subject (day 1, 8 h post-dose) spiked with 50 ng of **IV**, concentration of **II** equivalent to 37 ng/ml. The numbers in upper right hand corner correspond to peak heights expressed in arbitrary units.

Table 5
Concentration (ng/ml) of **I** and **II** in plasma of selected human subjects after a 25-mg oral dose of **I**

Subject	Analyte	Concentration (ng/ml)					
		0[a]	2	4	8	12	24
74	**I**	0	2.0	17.5	35.1	15.3	2.4
74	**II**	0	78.8	128.0	34.3	19.1	23.8
77	**I**	0	2.6	19.1	20.7	7.9	2.1
77	**II**	0	92.8	132.3	37.0	23.0	7.4

[a] Time after dosing (h).

compound or an internal standard especially at high concentrations of metabolites relative to the concentration of the analytes. The case presented in this paper clearly indicates the possibility of an interference of a metabolite M to the quantification of analyte **II**. A dramatic example of such an interference of metabolites to the quantification of an internal standard was observed in our laboratory during HPLC–MS–MS analyses of post-dose human urine samples after dosing with an oxytocin receptor antagonist [15]. In this case, the interference from metabolites was easily recognized by observing the unusually high increase in the area of internal standard peak in comparison with the peak area of the same amount of internal standard spiked into control or pre-dose urine. Contrary to the internal standard case, the detection of such an interference for the parent compound, without chromatographic separation, is difficult, and an additional effort in establishing the peak purity of the eluting peak of interest is necessary. This could be best accomplished by analyzing post-dose samples under gradient chromatographic conditions, and/or providing enough chromatographic retention of an analyte so that a potential metabolite(s) is well separated from the peak(s) of interest. The examples provided in this paper and in our previous communications [14,15] clearly illustrate the necessity of careful assessment of HPLC–MS–MS assay specificity in post-dose biological fluid samples in the presence of metabolites.

In conclusion, highly selective and sensitive assays for the determination of **I** and its carboxylic acid metabolite **II** in human plasma and semen were developed, but required different sample preparation procedures and different chromatographic conditions to eliminate the interference from a metabolite giving the response at the same MS–MS channel as used for the quantification of **II**.

Acknowledgments

The authors would like to thank Dr. J. Schwartz and Dr. K. Lasseter, the clinical monitor and clinical investigator, respectively, of the clinical trial from which biological fluid samples were available for analyses.

References

[1] P.J. DeSchepper, J. Imperato-McGinley, A. Van Hecken, I. DeLapeleire, A. Buntinx, J. Carlin, M.H. Gressi and E. Stoner, Steroids, 56 (1991) 469–471.
[2] R.L. Tolman, S. Aster, R.K. Bakshi, J. Bergem, H.G. Bull, B. Chang, G. Cimis, P. Durette, K. Ellswort, C. Esser, D.W. Graham, W.K. Hagmann, G.S. Harris, I. Kopka, T. Lanza, G.F. Patel, G.H. Rasmusson, S. Polo, S. Sahoo, J. Toney, D. Von Langen and B. Witzel, Eur. J. Med. Chem., 30 (suppl) (1995) 311–316.
[3] R.K. Bakshi, G.F. Patel, G.H. Rasmusson, W.F. Baginsky, G. Cimis, K. Ellsworth, B. Chang, H. Bull, R.L. Tolman and G.S. Harris, J. Med. Chem., 37 (1994) 3871–3874.
[4] G.J. Gormley, E. Stoner and R.C. Bruskewitz et al., N. Engl. J. Med., 327 (1992) 1185–1191.
[5] M. Ohtawa, H. Morikawa and J. Shimazaki, Eur. J. Drug Metab. Pharmacokinet., 16 (1991) 15–21.
[6] B.J. Gertz, D.Z. Wang, D.L. Ebel, S.J. Justice, K. Lasseter and J.I. Schwartz, Endocrine Society 77th Annual Meeting, June 14–17, 1995, P. 141.
[7] G. Harris, B. Azzolina, W. Baginsky, G. Cimis, G.H. Rasmusson, R.L. Tolman, C.R.H. Raetz and K. Ellsworth, Proc. Natl. Acad. Sci. USA, 89 (1992) 10 787–10 791.
[8] M.L. Constanzer, B.K. Matuszewski and W.J. Bayne, J. Chromatogr., 566 (1991) 127–132.
[9] M.L. Constanzer, C.M. Chavez and B.K. Matuszewski, J. Chromatogr. B, 658 (1994) 281–287.
[10] J.D. Glibert, T.V. Olah, A. Barrish and T.F. Gerber, Biol. Mass Spec., 21 (1992) 341–346.
[11] T.V. Olah, J.D. Gilbert, A. Barrish, T.F. Greber and D.A. McLoughlin, J. Pharm. Biomed. Anal., 12 (1994) 705–712.
[12] R.D. Amin, M.S. Schwartz and H. Cheng, Merck Research Laboratories, unpublished results.
[13] T.V. Olah, D.A. McLoughlin, M.J. Morris, R.A. Stearns and R. Miller, Merck Research Laboratories, unpublished results.

[14] B.K. Matuszewski, C. Chavez and M.L. Constanzer, 10th (Montreux) Symposium on Liquid Chromatography–Mass Spectrometry, Cornell University, Ithaca, New York, July 21–23, 1993.

[15] B.K. Matuszewski, M.L. Constanzer and C. Chavez, 12th (Montreux) Symposium on Liquid Chromatography–Mass Spectrometry, Hilton Head Island, South Carolina, November 1–3, 1995, manuscript in preparation.