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Development of high-performance liquid chromatography–tandem mass spectrometric methods for the determination of a new oxytocin receptor antagonist (L-368,899) extracted from human plasma and urine: a case of lack of specificity due to the presence of metabolites

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Abstract

The purpose of this work was to develop HPLC–MS–MS methods for the quantification of L-368,899 (**1**) in human plasma and urine and to evaluate the selectivity of these methods in post-dose samples in the presence of metabolites. Assays were based on double liquid–liquid extraction of the drug and internal standard (I.S., **2**) from basified plasma, evaporation of the extracts to dryness, derivatization of the primary amino groups of **1** and **2** with trifluoroacetic anhydride (TFAA) to form trifluoroacetylated (TFA) analogs, and HPLC analysis using tandem mass spectrometer equipped with the heated nebulizer interface as a detector. The derivatization with TFAA was required to eliminate the carryover and adsorption problems encountered when underivatized molecules were chromatographed, and allowed quantitation at low concentration (0.5 ng/ml) in plasma and urine. Initially, assays in control human plasma and urine were validated in the concentration range of 0.5–75 ng/ml, using simplified chromatographic conditions with a 2-min run-time and no separation of the drug from I.S.. Quantitation was based on the high selectivity of detection and multiple reaction monitoring (MRM) using the precursor→product ion combinations of m/z 651→152 and m/z 665→425 for the TFA-derivatized **1** and **2**, respectively. However, when selected post-dose urine samples from a clinical study were analyzed using this assay, the area of the I.S. peak was 4 to 7 times larger than the area of I.S. peak in pre-dose urines, indicating the presence of metabolites giving rise to the m/z 665→425 I.S. peak. A number of metabolites contributing to the I.S. ion pair were separated from **1** and **2** using a longer analytical column, a weaker mobile phase, and by extending the HPLC run-time to 12 min. Under these new conditions, the modified assays both in plasma and urine were validated in the concentration range of 0.5 to 75.0 ng/ml. These assays were selective in the post-dose urine samples in the presence of metabolites.

Keywords: Oxytocin; L-368,899

1. Introduction

Compound **1** [1-(((7,7-dimethyl-(2(*S*)-amino-4-methylsulfonyl)butyramido)-bicyclo[2, 2, 1]-heptan-

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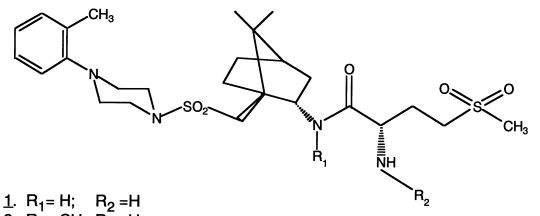

1. R_1= H; R_2 =H
2. R_1= CH_3;R_2 =H
3. R_1= H; R_2 =$COCF_3$
4. R_1= CH_3;R_2 =$COCF_3$

Fig. 1. Chemical structures of **1**, internal standard **2**, and their trifluoroacetylated analogs **3** and **4**, respectively.

1(*S*)-yl)methyl)sulfonyl)-4-(2-methylphenyl)- piperazine] (L-368,899, Fig. 1) was recently discovered as an orally bioavailable, non-peptide oxytocin antagonist with potential utility for managing preterm labor [1,2]. It was demonstrated that **1** inhibited postpartum oxytocin-stimulated uterine contractions, demonstrating that it is active in humans as an oxytocin receptor antagonist [3]. In order to support clinical pharmacokinetic studies with **1**, it was necessary to develop sensitive and specific methods for determination of **1** in human plasma and urine with the limit of quantification (LOQ) of less than 1 ng/ml. Due to the lack of good chromophore and poor absorbance of **1** in the ultraviolet (UV) region of the spectrum, the development of an assay based on high-performance liquid chromatography (HPLC) with UV absorption detection at subnanogram concentrations was not feasible. A method in plasma based on derivatization of the primary amino group of **1** with naphthalene dicarboxyaldehyde (NDA) in the presence of a nucleophile with the LOQ of 1 ng/ml was initially developed but required extensive sample clean-up both off-line and on-line using column switching, a careful selection of a nucleophile, and an automated precolumn derivatization [4]. The method had a limited assay range of 1–25 ng/ml and was applicable only to the assay of **1** in human plasma. In order to develop an alternative and more sensitive assay for **1** in both plasma and urine, a method based on HPLC with tandem mass spectrometric (MS–MS) detection was evaluated.

In the last four to five years, HPLC–MS–MS methodology has been demonstrated to be a powerful technique for quantitative determination of drugs and metabolites in biological fluids. Numerous bioanalytical methods have been developed using this method and the technique is currently considered as a method of choice for supporting clinical and preclinical pharmacokinetic studies [5–7]. We have reported extensively on several applications of this methodology [8–15]. It is generally believed that the application of HPLC–MS–MS for the determination of drugs and metabolites in biofluids practically guarantees specificity; sample preparation may be simplified or even eliminated, and none or very little chromatographic separation is required. Contrary to this common belief, a number of examples were recently presented illustrating the need for careful assessment of HPLC–MS–MS assay specificity including studies of matrix effect and ion suppression [16–18], evaluation of specificity in post-dose biological fluid samples in the presence of metabolites, instead of in control (blank) biofluids, and the need for an efficient extraction of analytes from biofluids and chromatographic separation [15,16]. In spite of the high selectivity of MS–MS detection, the coeluting and chromatographically unseparated metabolites can fragment to some extent in the atmospheric pressure chemical ionization (APCI) region of the mass spectrometer giving rise to the same ion as the one (i.e., protonated molecule) monitored for the parent compound and/or an I.S.. Further fragmentation of this ion in the collision cell of MS may lead to the formation of the same product ion as the one originating from the parent compound or an I.S.. This interference from a metabolite to the quantification of a parent compound was demonstrated by us earlier during determination of a 5α-reductase inhibitor in human plasma [15]. There is a potential for a similar interference of metabolites to the quantification of an internal standard (I.S.), especially when analogs rather than isotopically labeled compounds are used as internal standards. A striking example of this lack of specificity of the HPLC–MS–MS method due to the interference from metabolites to the quantification of an I.S. was uncovered during the development of an assay for **1** in human urine described in this paper. It was found that a number of metabolites present in post-dose urine samples and not chromatographically separated from the I.S. (**2**, Fig. 1) gave significant responses in the same channel (m/z 665→425) as used for monitoring the I.S.. The peak areas of the I.S. spiked at the same

concentration of 10 ng/ml were 4 to 7 times larger in post-dose urines than in pre-dose urine samples, indicating the presence of metabolites giving rise to the 665→425 I.S. peak. A number of metabolites contributing to the I.S. quantification were separated from **1** and the method was later modified requiring chromatographic separation of metabolites from **1**. The details of the HPLC–MS–MS assay methodology in control vs. post-dose biological fluid samples are presented and the need for careful evaluation of assay specificity in post-dose biological fluids in the presence of metabolites is emphasized. In addition, experiments required to demonstrate HPLC–MS–MS assay specificity in post-dose biological fluid samples are discussed.

2. Experimental

2.1. Materials

Compounds **1** and its *N*-methylated analog **2** (as a hydrochloride and maleate salt, respectively) were synthesized at Merck Research Laboratories (West Point, PA, USA). All solvents and reagents were of HPLC or analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The different lots of drug-free human heparinized plasma originated from Biological Specialties (Lansdale, PA, USA). Control human urine was available from laboratory personnel. Air (hydrocarbon-free), nitrogen (99.999%) and argon (99.999%) were purchased from West Point Supply (West Point, PA, USA). Trifluoroacetic anhydride (TFAA), trifluoroacetic acid (TFA), and formic acid (FA) originated from Aldrich (Milwaukee, WI, USA). Ammonium acetate (AA) was from Sigma (St. Louis, MO, USA).

2.2. Instrumentation

A Perkin-Elmer (PE) Sciex (Thornhill, Canada) API III tandem mass spectrometer equipped with a heated nebulizer (HN) interface, a PE I.S.S 200 autoinjector, and PE biocompatible binary pump (model 250) were used for all HPLC–MS–MS analyses. The data were processed using MacQuan software (PE Sciex) on a MacIntosh Quadra 900 microcomputer.

2.3. Chromatographic conditions

2.3.1. Control plasma and urine, HPLC system (I)

Chromatographic analyses were initially performed using a short BDS Hypersil C_8 (20×4.6-mm, Keystone Scientific, Bellefonte, PA, USA), 5-μm, analytical column and a mobile phase consisting of 90% acetonitrile (ACN) and 10% of the mixture of 10 m*M* AA in 0.1% FA in water, pumped at a flow-rate of 1 ml/min. The total run-time was 2 min. The retention times of the trifluoroacetylated derivatives **3** and **4** were about 0.3 min for both **3** and **4** and these derivatives were not separated from each other under these conditions. Both analytes were practically unretained on the analytical column (capacity factor, k', was about 0.6).

2.3.2. Post-dose plasma and urine, HPLC system (II)

Analyses were performed on a relatively long BDS Hypersil C_{18} (150×4.6 mm, Keystone Scientific) 5-μm, analytical column and a mobile phase consisting of 60% ACN and 40% of the mixture of 10 m*M* AA in 0.1% FA in water, protected with a BDS Hypersil C_{18} (20×4.6 mm, 5-μm) guard column pumped at a flow-rate of 1 ml/min. The total run-time was 12 min. Under these conditions the retention times of the trifluoroacetylated derivatives **3** and **4** were about 7.4 and 10.0 min, respectively, and they were baseline separated from each other. Both analytes **3** and **4** were effectively retained on the analytical column (capacity factor, k', was about 3.5 and 5.1 for **3** and **4**, respectively).

2.3.3. Post-dose plasma and urine, HPLC system (III)

The same analytical and guard columns as in system (II) were utilized but a stronger mobile phase consisting of 70% ACN and 30% of the mixture of 10 m*M* AA in 0.1% FA in water was employed. The flow-rate was 1 ml/min and the total run-time was 6 min. Under these conditions, the retention times of the trifluoroacetylated derivatives **3** and **4** were about 4.0 and 4.5 min, respectively. Both analytes were separated from metabolites under these conditions and the k' values were about 1.4 and 1.8 for **3** and **4**, respectively.

2.4. HPLC–MS–MS conditions

A PE Sciex triple quadrupole mass spectrometer (SCIEX API III) was interfaced via a Sciex HN probe with the HPLC system. The HN probe was maintained at 500°C and gas phase chemical ionization was effected by a corona discharge needle (+4 μA) using positive ion atmospheric pressure chemical ionization (APCI). The nebulizing gas (air) pressure was set for the HN interface at 80 psi. The auxiliary flow was at 2.0 l/min, the curtain gas flow (nitrogen) was at 0.9 l/min, and the sampling orifice potential was set at +70 V. The dwell time was 400 ms, and the temperature of the interface heater was set at 60°C. Q1 and Q3 were operated at unit mass resolution. The mass spectrometer was programmed to admit the protonated molecules $[M+H]^+$ at m/z 651 for **3** and m/z 665 for **4** via the first quadrupole filter (Q1), with collision-induced fragmentation at Q2 (collision gas argon, 400×10^{13} atoms cm^{-2}), and monitoring the product ions via Q3 at m/z 152 and 425 for **3** and **4**, respectively. The electron multiplier setting was −3.3 kV. Peak area ratios obtained from selective reaction monitoring of analytes (m/z 651→152)/(m/z 665→425) were utilized for the construction of calibration curves, using weighted ($1/y$) linear least-square regression of the plasma or urine concentrations and the measured peak area ratios. Data collection, peak integration and calculations were performed using MacQuan PE-Sciex software.

2.5. Standard solutions

A stock solution of **1** (equivalent to 1 mg/ml of a free base) was prepared in acidified methanol containing one drop of 1 *M* HCl in 10 ml of methanol. This solution was further diluted with acidified methanol to give a series of working standards with the concentrations from 0.005 to 0.75 μg/ml. The internal standard **2** was also prepared as a stock solution (1 mg/ml of the free base) in the acidified methanol. This solution was further diluted with methanol to give a working standard of 0.1 μg/ml which was used for all analyses. All standards were prepared once a month and stored at 5°C.

2.6. Sample preparation and derivatization

A 1-ml aliquot of plasma or urine was pipetted into a 15-ml centrifuge tube and 100 μl of the working standards of **1** and 100 μl of the working standard of **2** (equivalent to 10 ng/ml) were added followed by the addition of 1 ml of 0.05 *M* carbonate buffer (pH 9.8). After addition of 5 ml of methyl-*tert*-butyl ether (MTBE), the tubes were capped with Teflon-lined caps, the mixture was mixed and rotated for 15 min, centrifuged, and the organic layer was transferred to a clean 15-ml centrifuge tube. The liquid–liquid extraction was repeated by adding a second 5-ml volume of MTBE to the original tube, vortex-mixing, centrifugation, and removal of the organic layer to the tube containing the initial MTBE extract. Before evaporation of the combined organic phases to dryness, 0.5 ml of MTBE solution containing 0.5% TFA was added to prevent adsorption of **1** and **2** to the walls of the centrifuge tube. The organic extract was evaporated to dryness under a stream of nitrogen at 50°C, and 100 μl of neat TFAA was added, the tubes were capped, mixed by vortex, and the mixture was allowed to react for 15 min at room temperature. The excess of derivatizing reagent was evaporated to dryness under a stream of nitrogen at room temperature, the residue was reconstituted in 125 μl of mobile phase and, after vortexing, sonication, and centrifugation for 10 min at $2050\times g$, the liquid layer was transferred to the glass inserts which were loaded on the autosampler. Fifty μl of the residue was injected onto the HPLC–MS–MS system.

2.7. Precision, accuracy, and recovery

The precision of the method was determined by the replicate analyses ($n=5$) of human plasma and/or urine containing **1** 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 internal standard versus drug concentration. The unknown sample concentrations were calculated from the weighted least-squares regression analysis of the standard curve. The standard curve samples were prepared and assayed daily with quality control and unknown samples. The

accuracy of the method was expressed by [(mean observed concentration)/(spiked concentration)]× 100. The recovery was determined by comparing the peak area of **1** extracted from plasma or urine to that of standards derivatized and injected directly.

3. Results and discussion

Various approaches to the determination of **1** in human plasma and urine were initially considered and included a method based on HPLC with UV, electrochemical (EC), and fluorescence (FLU) detection. Sensitivity of UV detection was very poor due to the absence of a highly absorbing chromophore in the molecule of **1**. Also, the oxidation potential of **1** was rather high (+1.0 V) making EC detection inadequate for high sensitivity detection in highly complex matrices. The HPLC–FLU approach was most successful and was based on chemical derivatization of the primary amino group of **1** with NDA in the presence of a nucleophile to form highly fluorescent benzo[*f*]isoindole derivatives. Using this method, an assay in plasma with the LOQ of 1 ng/ml was developed [4] but required an extensive sample clean-up before derivatization, relatively long analysis time, an automated precolumn derivatization, a column switching, and a very careful choice of a nucleophile to achieve an adequate derivatization yield. Therefore, in order to develop efficient and sensitive assays both in plasma and urine with the LOQ of less than 1 ng/ml, HPLC–MS–MS approach was evaluated.

Initially, an attempt was made to adapt the HPLC method for determination of **1** in dog plasma developed to support preclinical studies at relatively high concentrations of ~5 ng/ml [19]. This method was based on MS–MS detection of underivatized **1**. However, when this method was used for analysis of human plasma and urine at low concentrations, severe adsorption and carryover effects were noted in our HPLC system, probably due to the presence of a primary amino group in the molecule of **1**. These effects are quite common for compounds containing amino groups and are usually exhibited at low <5 ng/ml) concentrations. In order to eliminate these carry-over and adsorption effects, the primary amino groups of **1** and the internal standard **2** (a *N*-methylated analog of **1**) were acetylated with TFAA to form trifluoroacetylated analogs **3** and **4** (Fig. 1). After derivatization, the adsorption and carry-over effects were effectively eliminated at low analyte concentrations, allowing quantification at concentrations below 1 ng/ml.

3.1. Development of HPLC–MS–MS assays in control plasma and urine

The product-ion mass spectra of the trifluoroacetylated derivatives **3** and **4** of **1** and **2** are shown in Fig. 2.

The intense product ions at m/z 152 and 425 for **1** and **2**, respectively, were chosen for quantification in the multiple reaction monitoring (MRM) mode. Using the precursor→product ion combinations of m/z 651→152 and 665→425 for drug and I.S., respectively, an assay in control plasma and urine was initially developed. Simplified chromatographic conditions [HPLC system (I)] with a two-min run time and no separation of drug and I.S. were utilized in this initial assay. The extraction method was a double liquid–liquid extraction from buffered (pH 9.8) plasma or urine with MTBE.

After derivatization with TFAA, the trifluoroacetylated analogs of both **1** and I.S. (**2**) were monitored by MS–MS. The representative chromatograms in plasma are shown in Fig. 3.

The assays in control plasma and urine were validated in the concentration range of 0.5 to 75 ng/ml (Table 1). The limit of quantification (LOQ) was 0.5 ng/ml both in plasma and urine and was defined as the lowest concentration on the standard curve for which precision was better than 10% and accuracy was within 15% of the spiked concentration. The representative equation for the standard line in plasma, for example, was $y=0.0879x+0.0139$, with the correlation coefficient of 0.9992.

Quality control (QC) samples were prepared at nominal concentrations of 2 and 60 ng/ml and stored at −20°C. Duplicate QC samples were subjected to three freeze-thaw cycles and assayed. The data obtained indicated that **1** was stable during three consecutive freezing and thawing steps (data not shown). In addition, five separate standard lines were

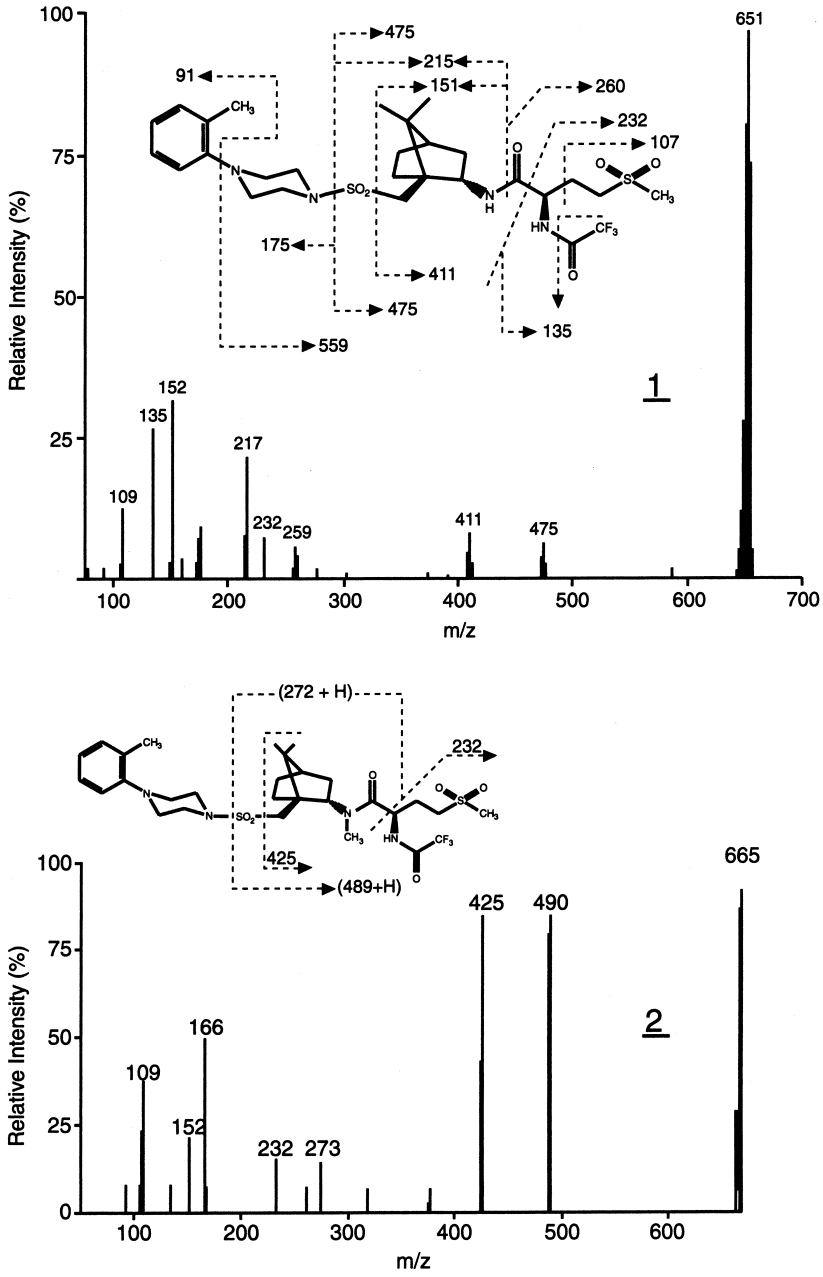

Fig. 2. Product-ion mass spectra of the protonated molecules of the trifluoroacetylated analogs of **1** at $[M+H]^+=651$ and **2** at $[M+H]^+=665$.

constructed in five different human plasma lots to assess both the efficiency of extraction from different plasma lots and a potential matrix effect during MS–MS analysis. The coefficient of variation (C.V.) of the slopes of these lines was 4.4%. The absolute peak areas of **1** and **2** were also similar between different plasma lots indicating that ion suppression, if any, was practically the same in different plasmas.

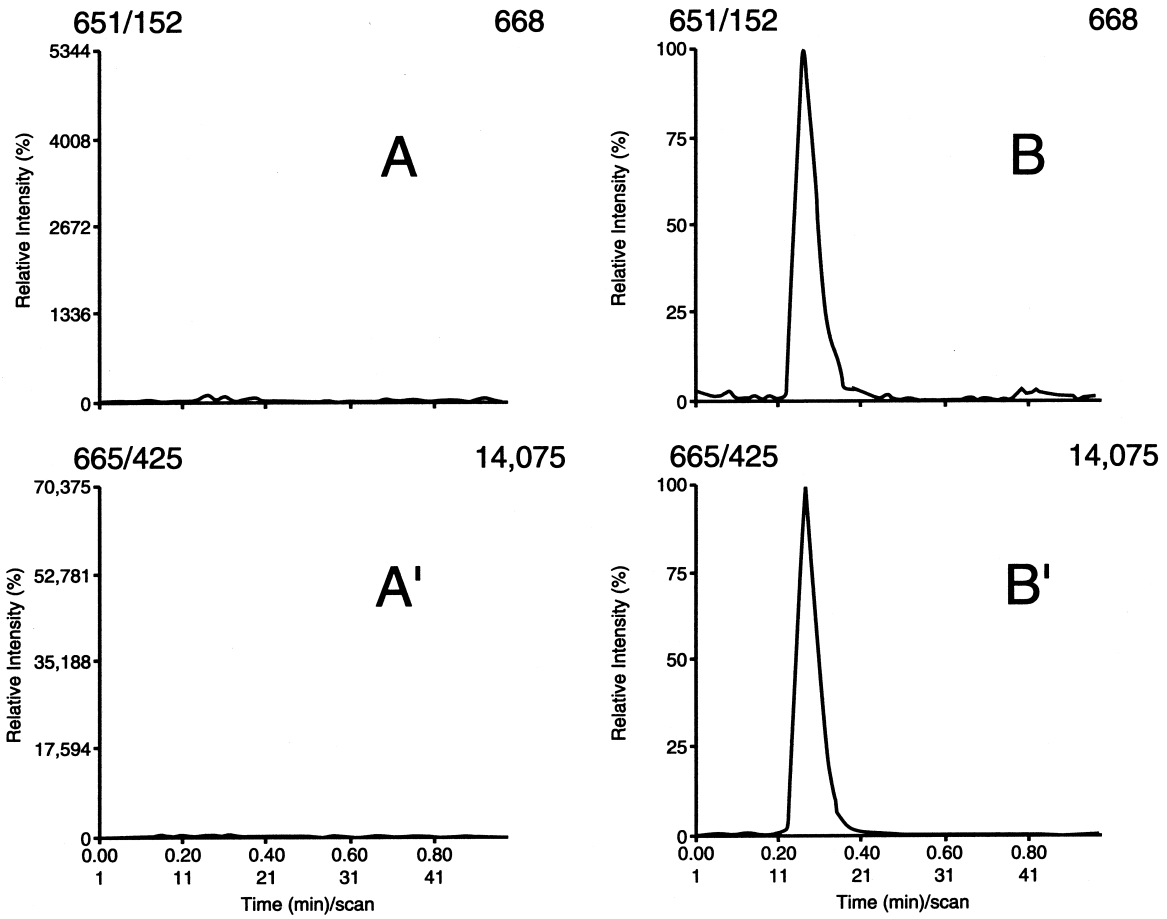

Fig. 3. Representative LC/MS–MS chromatograms of plasma (1 ml) extracts obtained by multiple reaction monitoring at m/z 651→152 (channel 'a') for **1** and m/z 665→425 (channel 'b') for internal standard **2**; (A, A′)-blank control plasma monitored at channels 'a' and 'b' respectively; (B, B′)-control plasma spiked with 0.75 ng/ml of **1** and 10 ng/ml of **2** and monitored at channels 'a' and 'b' respectively. The numbers in the upper right hand corner correspond to peak heights expressed in arbitrary units.

3.2. Analyses of post-dose plasma and urine samples

The method validated in control plasma and urine was utilized for the analyses of more than 300 plasma samples and selected urine samples from a single dose oral safety and tolerability study in humans dosed with **1**. The areas of I.S. spiked into control plasma monitored during the validation experiment and in post-dose plasma samples were practically the same. In addition, approximately the same I.S. peak area was observed during validation of the assay in control urine. However, an examination of the peak areas of the internal standard in urine samples after oral dosing with 500 mg of **1** indicated that in post-dose urines the area of I.S. was 4–7 times larger than the peak area of the same amount of I.S. (10 ng) spiked into control urine (1 ml). In addition, the peak area of I.S. in post-dose urines was highly variable. Representative peak areas of I.S. spiked into control urines and in selected post-dose urine samples are shown in Table 2.

In order to explain this significant increase in the area of I.S. in post-dose urines, it was assumed that this rise was due to the presence of metabolites co-eluting with the I.S. which was practically unretained on the HPLC column and eluted at about 0.3 min (k'~0.6). In addition, these metabolites were

Table 1
Precision[a] and accuracy[b] data of the determination of **1** in control plasma and urine using HPLC–MS–MS

Nominal concentration (ng/ml)	Accuracy (%)		Precision C.V.(%)	
	Plasma	Urine	Plasma	Urine
0.50	98	86	6.0	8.9
0.75	96	99	4.6	3.0
1.0	96	96	6.4	7.5
2.5	104	104	2.8	2.1
5.0	102	103	4.3	3.2
10.0	99	108	8.4	9.2
25.0	100	100	3.2	5.4
50.0	97	95	1.2	4.5
75.0	98	97	3.7	3.0

[a] Expressed as coefficient of variation ($n=5$).
[b] Calculated as a [(mean calculated concentration)/(spiked concentration)]×100.

detected in the same MS–MS channel (m/z 665→425) as used for monitoring the I.S.. This hypothesis was confirmed experimentally by performing chromatographic separation of I.S. from metabolites under gradient HPLC conditions and later utilizing separation under highly efficient isocratic conditions (HPLC system II, see Section 2). A dramatic illustration of the presence of a number of metabolites giving rise to the same m/z 665→425 pair as for the I.S. is presented in Fig. 4.

At least seven early eluting peaks giving rise to the same m/z 665→425 ion pair as used for monitoring the I.S. were detected. The I.S. (t_R~10 min) was clearly separated here from both **1** (t_R~8 min)

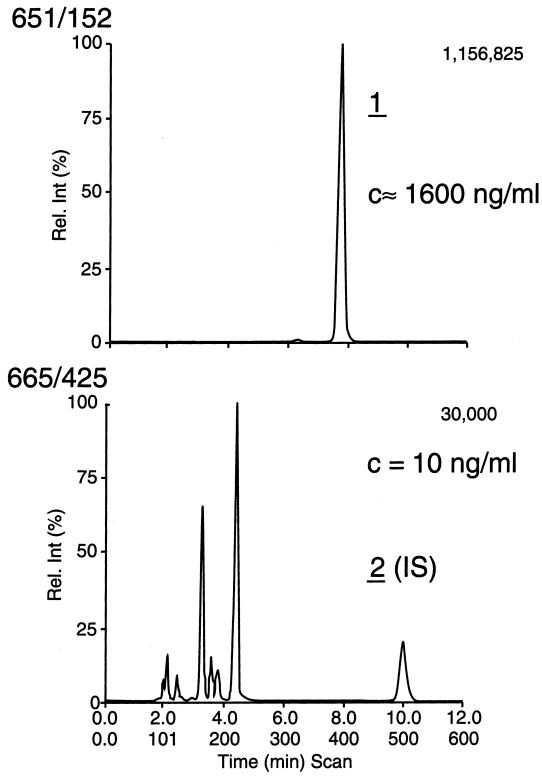

Fig. 4. Representative MS–MS chromatograms of the pooled post-dose urine extract (0–6 h) of a subject participating in a single dose safety and tolerability study after dosing orally with 500 mg of **1**, and after HPLC separation of the TFA-derivatized **1** and the internal standard **2**; Total run-time was 12 min, and the HPLC system II (see Section 2) was used for analysis. The numbers in the upper right hand corner correspond to peak heights expressed in arbitrary units. TFA-derivatized **1** and **2** were monitored at m/z 651→152 and 665→425, respectively.

Table 2
Peak areas of the derivatized internal standard (**2**) spiked at 10 ng/ml to control human urine and to the post-dose urines of a subject dosed orally with 500 mg of **1**

	Peak area of internal standard[a]
Control urine	66 304[b]
Post-dose urine[c]	
0–2	300 499
2–4	481 971
4–8	263 219

[a] In arbitrary units.
[b] Mean value ($n=45$) from validation experiment in control urine; urine samples from five different subjects were spiked and analyzed ($n=9$ for each urine), S.D.=±7028 (C.V.=10.6%).
[c] Collection interval post-dose (h).

and metabolites (t_R~2–5 min). Without chromatographic separation, these early eluting metabolite peaks coeluted with the I.S. resulting in the highly elevated peak areas for I.S. in post-dose urines in comparison with control urines. If analyses in post-dose urine samples were performed without chromatographic separation (as in Fig. 3) the concentrations of **1** in these samples would be significantly underestimated because peak area ratios of **1**/**2** were used as a measure of drug concentration.

The presence of a number of metabolites giving the same ion at m/z 665 in the chemical ionization region of the MS–MS system as for the I.S., is also illustrated in Fig. 5. A total ion chromatogram (Q1

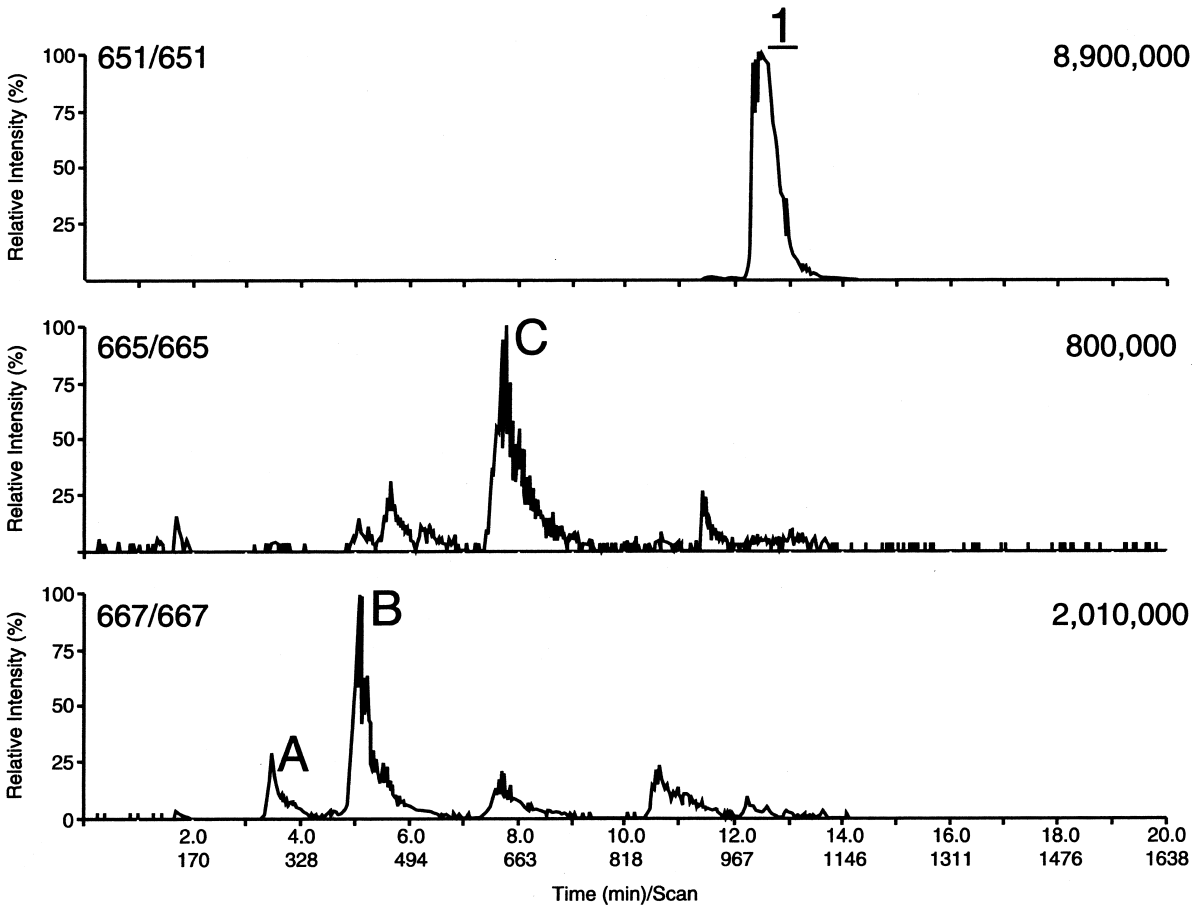

Fig. 5. Extracted mass chromatograms (Q1 scan) at m/z 651 (for **1**), 665 and 667 (for monohydroxylated metabolites of **1**) obtained from total ion chromatograms (TIC) of post-dose urine sample of a human subject dosed with 500 mg of **1**; Gradient chromatographic conditions were utilized here (6 min at 40% ACN–60% 10 m*M* AA in 0.1% FA, followed by a gradient to 90%/10% in 10 min, and 4 min at 90% ACN–10% 10 m*M* AA in 0.1% FA; column: BDS Hypersil C_8 (50×4.6 mm, 5 μm); flow-rate 1 ml/min,); Urine sample was not spiked here with **2**; Pre-dose urine sample of the same subject (chromatogram not shown) did not indicate the presence of any peaks in all three channels monitored; The numbers in the upper right hand corner correspond to peak heights expressed in arbitrary units.

scan, chromatogram not shown) indicated the presence of at least four new peaks, in addition to the TFA-derivatized parent compound (t_R 12.6 min), all of them eluting with t_R shorter than the parent compound. The most intense ions obtained from the Q1 profiles of these peaks were at 667, 665, 665, and 667. Some of these peaks were broad and constituted a not fully separated mixture of compounds. The extracted mass chromatogram at m/z 665 illustrating the presence of various metabolites exhibiting the m/z 665 $(M+H+14)^+$ peaks similar to the I.S. was compared with the extracted mass chromatogram at m/z 651 (protonated TFA-derivatized parent compound) and with the extracted mass chromatogram at m/z 667 corresponding to the monohydroxylated species (Fig. 5). Again, a number of metabolites with the m/z 665 were observed including a major peak **C** (Fig. 5).

The Q1 spectrum (Fig. 6C′) of the major peak **C** from Fig. 5 at t_R 7.8 min clearly indicated the presence of a metabolite with the molecular weight 14 mass units higher $(M+H)^+=665$ than the TFA-derivatized **1** at $(M+H)^+=651$. The product-ion mass spectrum of the m/z 665 peak (Fig. 6C)

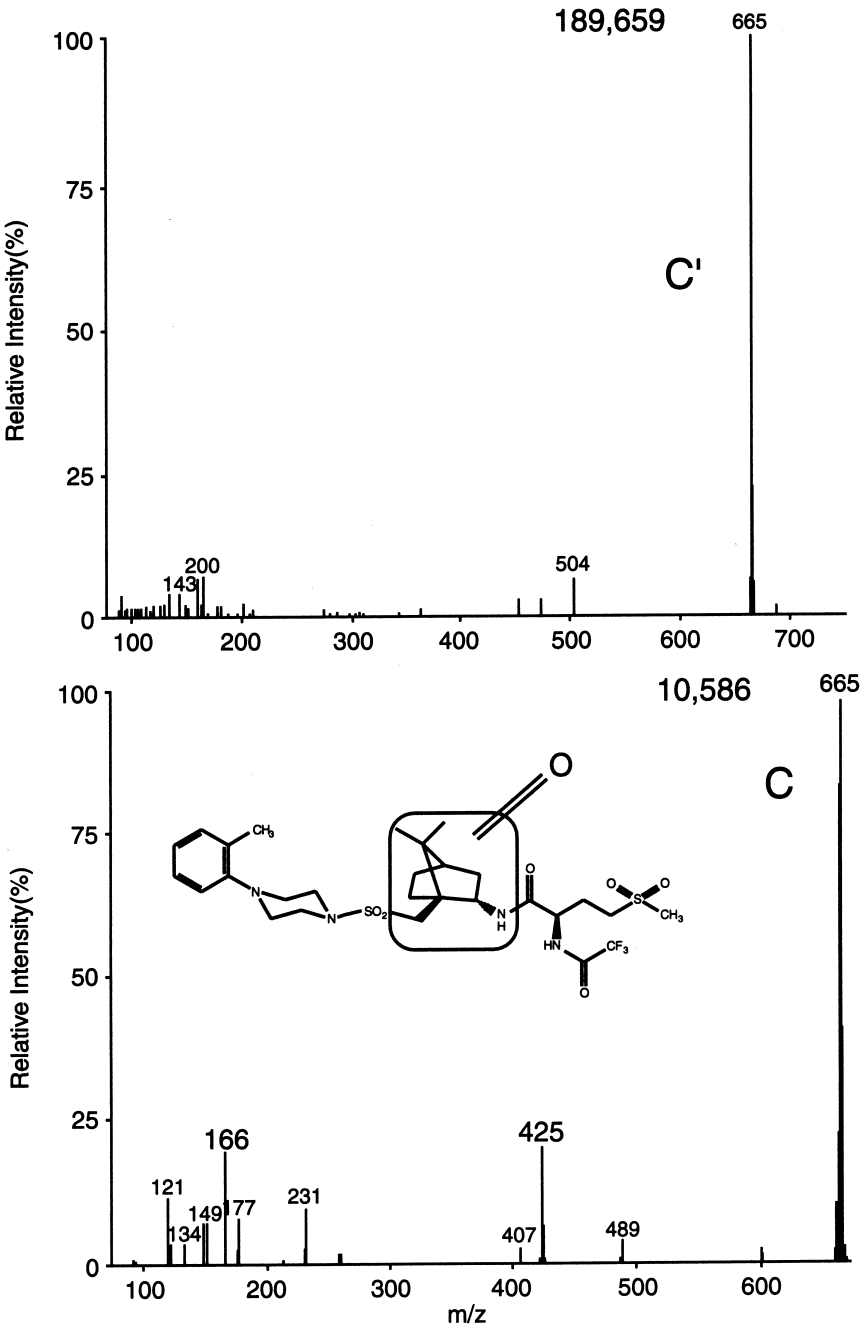

Fig. 6. Q1 spectrum (**C**′) of the major metabolite C (see Fig. 5) of **1**, and the product-ion mass spectrum (**C**) of the protonated molecule of metabolite C at *m*/*z* 665.

indicated the presence of two major fragments at *m/z* 425 and 166 which are indicative of the replacement of one of the methylene (or a methyl group) in the camphor moiety of **1** with the carbonyl or an aldehyde group, respectively. This assessment (see below) was based on the comparison of the product-ion mass spectra of monohydroxylated metabolites **A** and **B** in Fig. 7 with **1** (Fig. 2) and with the

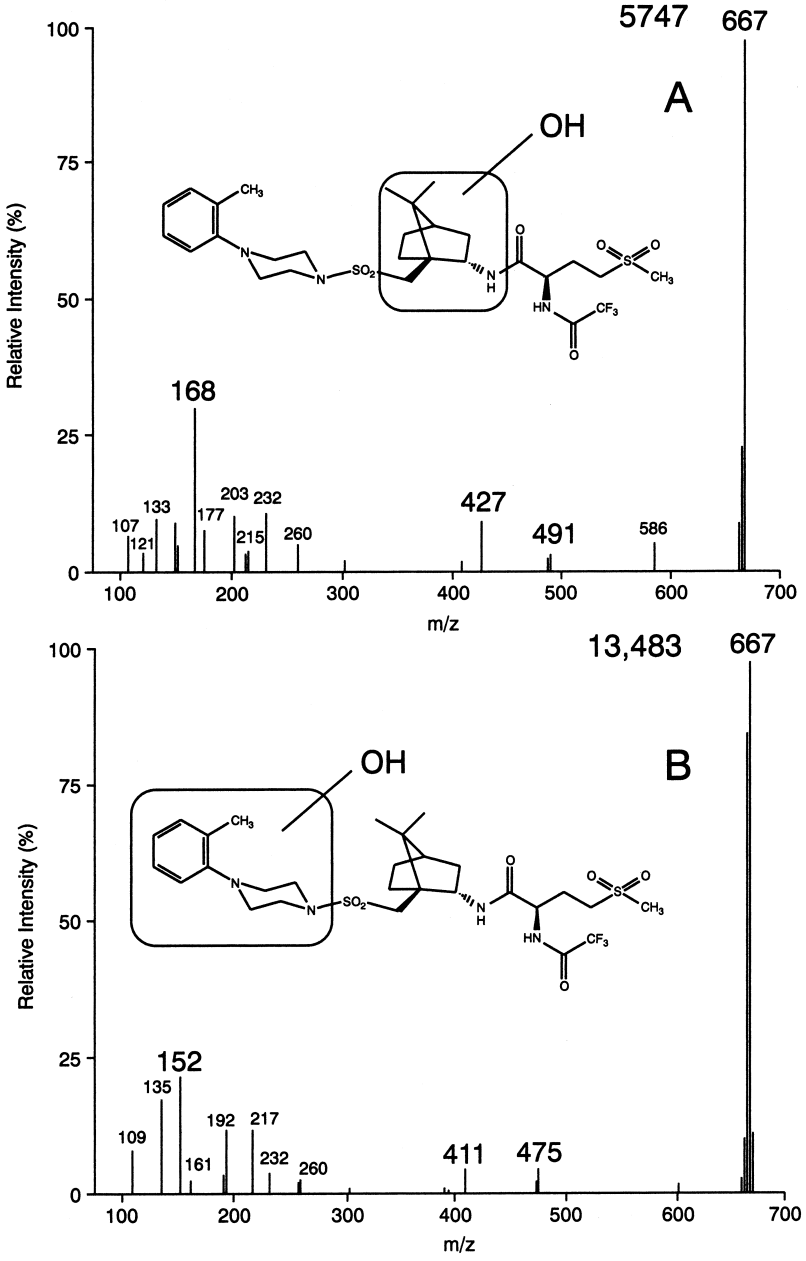

Fig. 7. Product-ion mass spectra of the molecular ions at *m/z* 667 of the TFAA-derivatized monohydroxylated metabolites A and B of **1** present in post-dose urine samples of a subject dosed with 500 mg of **1**; For comparison with the product-ion mass spectrum of the protonated molecule of **1**, see Fig. 2.

product-ion mass spectrum of **C** (Fig. 6C). When metabolite **C** was not chromatographically separated from the I.S., it gave rise to the response at *m/z* 665→425 used for monitoring the I.S..

In addition to more than six monohydroxylated metabolites, at least six other metabolites including dihydroxylated analogs were also detected. The unequivocal determination of the structure of all these metabolites was not attempted and will require further studies including comparison of the product ion MS and NMR spectra of the chromatographically isolated peaks with authentic samples, and chiral analyses of the reaction products, the latter due to a very rich stereochemistry involved in the formation of hydroxylated species on the camphor and 2-methylphenylpiperazine rings.

The molecular ions and the chemical structures of the major monooxygenated metabolite peaks **A**, **B** and **C** (Fig. 5) was tentatively assessed by taking the parent scans of the major fragments originating from these peaks, followed by the product ion spectra (MS–MS spectra) of the corresponding molecular ions. Parent ion scans of **A** and **B** indicated that the protonated molecules for both of these metabolites were at *m/z* 667, whereas for **C** were at *m/z* 665. Product-ion spectra of the protonated molecules of **A** and **B** at *m/z* 667 (Fig. 7) clearly indicated that in the case of **A**, the monohydroxylation has occurred on one of the carbon atoms of the camphor moiety, whereas in the case of **B** on the 2-methylphenylpiperazine moiety of **1**.

This assessment was made by comparing the product-ion spectra of **A** and **B** (Fig. 7) with **1** (Fig. 2). The presence of fragments at *m/z* 491, 427 and 168 in spectrum **A**, which were absent in **B** and in **1** (Fig. 2), indicated that oxygenation has occurred on the camphor ring of **1**. By comparison, the presence of fragments at *m/z* 475, 411, 152 and especially at 192, and lack of 491, 427 and 168 in spectrum **B** indicated that monohydroxylation has occurred on a 2-methylphenylpiperazine side of the molecule, as indicated in Fig. 7. Further oxidation of the monohydroxylated metabolite **A** may have led to the formation of metabolites similar to **C** (Fig. 5 and 6) containing a carbonyl group, instead of the hydroxyl group, on the camphor ring of **1**. There are four methylene carbons and two methyl groups in the camphor moiety of **1** which may be potentially oxidized to the corresponding ketones or aldehydes from the monohydroxylated metabolites similar to **A**. These carbonyl-containing metabolites, with the protonated molecules at *m/z* 665, upon fragmentation in Q2 gave the same product ion at *m/z* 425 (Fig. 6C) as observed for the I.S. (Fig. 2). With no chromatographic separation, all these monocarbonylated metabolites analogous to **C** (Fig. 6C) would give rise to the I.S. peak at *m/z* 665→425. The presence of the same metabolites as those shown in Figs. 4 and 5 was also confirmed by direct analyses of post-dose urine extracts without derivatization with TFAA, but these data will not be presented here.

The presence of metabolites interfering with the quantification of **2** was not apparent in post-dose plasma samples, probably due to the lower concentrations of metabolites in plasma and the need for dilution of plasma samples to determine **1** in the relatively narrow assay concentration range of 0.5 to 75 ng/ml. In order to avoid any interference from metabolites to the determination of **1** and **2**, both assays in plasma and urine were modified to include an effective chromatographic separation step of metabolites from **1** and **2**.

3.3. Modified assays in plasma and urine

The assays in both plasma and urine were revalidated under more efficient chromatographic separation conditions (HPLC system II) and after separation of **1** from I.S. and interfering metabolites. The capacity factor (k') was increased from 0.3 for both **1** and **2** (HPLC system I) to 3.5 and 5.1 (HPLC system II), respectively. The precision of the assay in plasma, for example, was better than 9% C.V., and accuracy was in the range of 90 to 114% at all concentrations within the standard curve range of 0.5 to 75 ng/ml. These assays were later modified even further using HPLC system III to shorten the run-time from 12 min to 6 min and to increase the assay concentration range in plasma from 1 to 500 ng/ml, and in urine from 1 to 1000 ng/ml. Under these modified HPLC conditions (HPLC system III), separation of metabolites from **1** and **2** was also provided.

3.4. Comments about the need for assessment of specificity of HPLC–MS–MS assays in biofluids in the presence of metabolites

The example presented in this paper clearly indicated the need for careful assessment of assay specificity in post-dose biological fluid samples as opposed to validations performed in control biofluids. In the case presented here, the metabolites interfered with the quantification of an internal standard, a *N*-methyl analog of **1**. In the case reported by us earlier [15], a metabolite interfered with the quantification of a parent drug. It is much easier to detect the interference of metabolites to the quantification of I.S. than a similar interference to the determination of a parent drug, by careful examination of the absolute peak areas (heights) of the I.S. peak in control vs. post-dose biological fluid samples. An unusual and significant increase in the I.S. peak size may be indicative of the presence of metabolites not separated from the I.S. but detected in the same MS–MS channel as used for monitoring the I.S.. Without chromatographic separation, such determination for the parent compound in post-dose samples is impossible. Therefore, it is advisable to analyze pooled post-dose samples under significantly more efficient chromatographic conditions (a gradient or an isocratic conditions with an increased k') to assess if there are any new peaks which are separated from the analytes of interest and detected at the same MS–MS channels as used for monitoring the analytes.

3.5. Comments about the importance of choosing an appropriate internal standard in HPLC–MS–MS assays in biofluids

The example shown in this paper clearly illustrate the potential pitfalls in using an internal standard, a *N*-methyl analog of an analyte **1**, monitored in the MS–MS channel in which, as it was later discovered, the response from metabolites present in post-dose samples was also observed. Without chromatographic separation of I.S. from metabolites, erroneous concentration data for the analyte would have been obtained.

The following should be taken into account before an I.S. is chosen for the HPLC–MS–MS assays:

- Whenever possible, a stable isotope-labeled (SIL) molecule of the analyte should be utilized. The major reason for using the SIL I.S. is to eliminate the matrix effect observed especially when a turbo ion spray interface (TISP) is utilized. This aspect of HPLC–MS–MS assay specificity was discussed earlier in separate papers [16–18]. The availability of the SIL I.S. is usually limited to the parent compound, and such SIL internal standards are not available for multi-component analyses of metabolites. The isotopic purity of a SIL I.S. is of great importance to avoid any ‘cross-talk’ between the MS–MS channels used for monitoring the parent compound and the I.S.. This isotopic purity is especially important when high sensitivity assays for parent compound (LOQ$<$0.5 ng/ml) are required.
- When an analog rather than a SIL I.S. is used, in addition to evaluating the ‘cross-talk’ effect between channels used for monitoring the analyte(s) and I.S., the assessment of specificity in post-dose samples under efficient chromatographic conditions is required. Also, the assessment of the matrix effect, especially when none or little chromatographic separation is provided, is necessary, since the matrix effect may be different for an analyte(s) and the I.S.. The analogs with the molecular weights similar to the potential metabolites should be avoided.
- Whenever possible, an efficient extraction from biofluids and chromatographic separation (large k') should be provided for all analytes and the I.S.. This ‘classical’ separation approach is still the most reliable for obtaining high quality analytical data no matter if MS–MS rather than conventional detection methods are used in quantitative determination of drugs and metabolites in biofluids based on HPLC.

In conclusion, the sensitive and specific HPLC–MS–MS methods for the determination of **1** in human plasma and urine were developed but required a number of modifications in comparison with methods developed initially in control biofluids due to the interference of metabolites with the quantification of an internal standard. The need for careful assessment

of assay specificity in post-dose biological fluids in the presence of metabolites was emphasized and some experiments required to demonstrate HPLC–MS–MS assay specificity in post-dose biological fluid samples were presented. In addition, the proper choice of internal standards for HPLC–MS–MS analyses was highlighted.

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