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Liquid chromatographic-tandem mass spectrometric urine assay for a highly metabolized cyclic ureidobenzenesulfonamide: issues concerning assay specificity and quality control preparation

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Abstract

An LC–MS–MS method was validated for the quantitation of a β_3 agonist (**A**) in human urine to support Phase I studies. **A** was designed to accelerate metabolism for weight reduction. During assay development a significant loss of **A** was apparent from frozen urine quality control samples. The addition of 0.75% bovine serum albumin (BSA) in urine (v/v) was required to maximize the recovery of **A** from urine. Urine samples were basified and extracted into methyl *t*-butyl ether–isopropyl alcohol (90:10, v/v). The organic layer was washed, evaporated, reconstituted, and injected onto a 5 cm, C8 HPLC column prior to MS–MS analysis. The standard curve was linear from 5 to 500 ng/ml. Intraday precision for peak area ratios from BSA urine samples at seven separate concentrations over a range of 5–500 ng/ml ($n = 5$) was $< 4.0\%$ and calculated concentrations were within 91–115% of nominal concentrations. Interday precision for BSA urine quality control (QC) samples at four separate concentrations ($n = 10$ of each) was $< 5.0\%$ and individual calculated concentrations were within 90–111% of nominal concentrations. This work emphasizes that potential metabolites and quality control standards should be prepared and assayed as early as possible in method development, especially before the sample collection section of the clinical protocol is prepared. The methods described here have wide utility to other compounds containing basic benzene sulfonamides and to β_3 agonist candidates.

Keywords: LC/MS/MS; Liquid–liquid extraction; β_3 agonist

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1. Introduction

Potent and selective pyridylethanolamine β_3-adrenergic receptor agonists, one of which is compound **A**, a cyclic ureidobenzensulfonamide (Fig. 1), were developed for the treatment of obesity through the mechanism of stimulating lipolysis in white and brown adipose tissue [1–5].

Early in clinical assay development, preclinical studies suggested **A** was extensively metabolized in species other than humans. Based on metabolism of **A**, three alcohol and one ketone analog of **A** were synthesized (**B**–**D**, Fig. 1). These analogs are identical in molecular weight and chemical composition to metabolites found in other species, however, position of oxidation may differ between analogs and metabolites. An analog of **A,** identical to a free amine metabolite, was also synthesized (**E**, Fig. 1). Plasma and urine assays were required to determine the pharmacokinetics and metabolism of **A** in man to support Phase I clinical trials for clinical development. Plasma and urine assays were identical to one another but the work described here focuses on the urine assay.

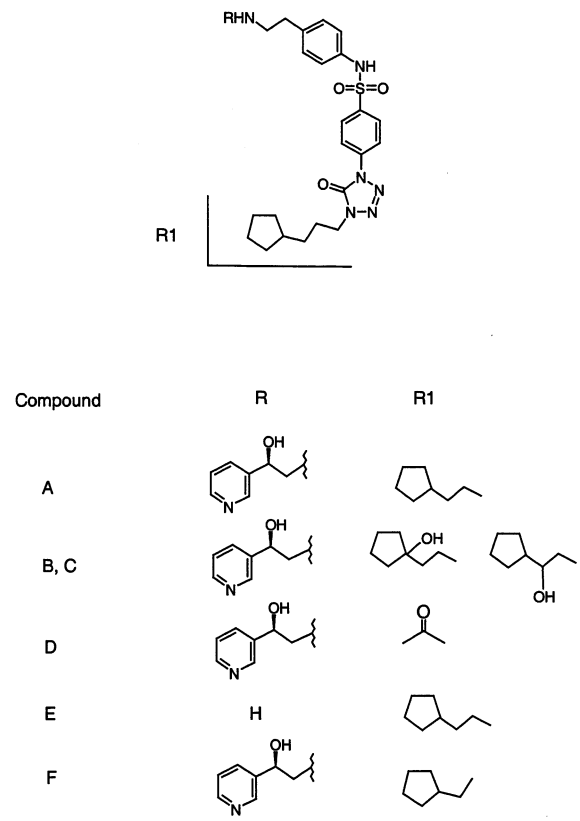

Fig. 1. Structures of β_3-agonist (**A**) two monohydroxylated analogs of **A** (**B** and **C**), a ketone analog (**D**), amine analog (**E**) and the internal standard (**F**).

An HPLC-UV absorbance assay was successfully validated for **A** from five separate sources of control urine. When synthetic analogs of **A** became available, suspected to be similar or identical to human metabolites, neat solutions of these analogs were chromatographed to determine specificity of the assay. The result was baseline separation of alcohol and ketone analogs (**B**–**D**) from the parent drug and internal standard **F** (IS), but co-elution of parent **A** with its suspected free amine metabolite **E** (Fig. 2). Co-elution of **A** and **E** occurred despite attempts to resolve the two using several different C18 and C8 HPLC columns. The columns investigated were Hypersil, YMC, Zorbax, Waters. The mobile phases used with these columns were composed of acetonitrile and one of the following aqueous components at or around pH 3.0: ammonium formate, ammonium acetate, both ammonium formate and acetate, TFA. Thus, early in clinical assay development, it became clear that a mass spectral technique would be needed to specifically quantitate **A** in plasma and urine. Months ahead of the study, it was also found there was low recovery of **A** from frozen urine quality control samples. The assay was further modified to eliminate low recovery of **A** from urine.

This work demonstrates the need to investigate specificity and to analyze quality controls early in assay development. The methods described here have wide utility to other compounds containing basic benzene sulfonamides. Some of these have been investigated as separate β_3 agonist candidates [1–5], that are highly metabolized and show some of the same recovery issues from frozen urine.

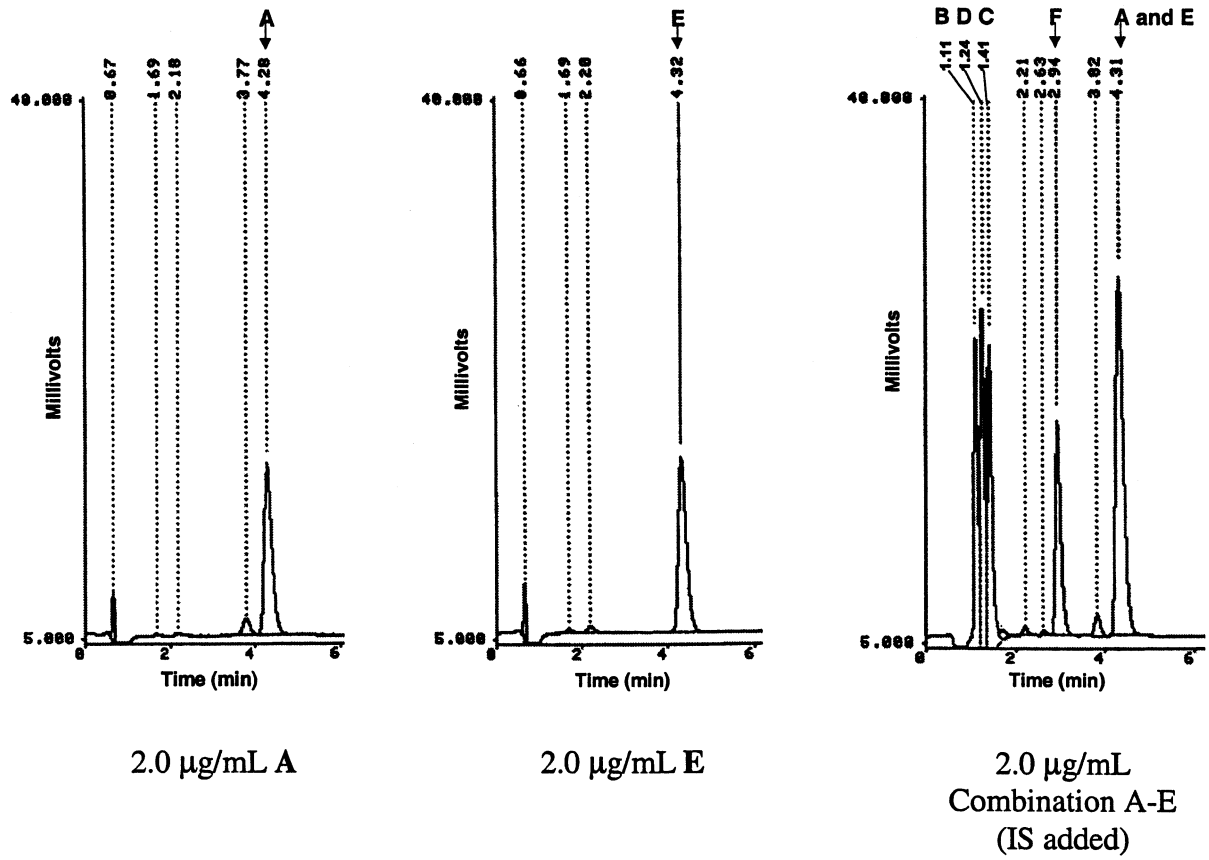

Fig. 2. HPLC-UV chromatograms of neat solutions of **A** and suspected urinary metabolites for assay specificity.

2. Experimental

2.1. Chemicals and reagents

A, **B**, **C**, **D**, **E** and **F** (IS) were synthesized at Merck Research Laboratories, Merck & Co. (West Point, PA) [1] and used, as received. A commercial 35% solution of bovine serum albumin (BSA), stabilized with sodium chloride and preserved with sodium azide, was purchased from Sigma (St. Louis, MO) and used to block the adsorption of **A** to urine collection containers. The following reagents were obtained from Fisher Scientific (Pittsburgh, PA): (1) optima grade acetonitrile, isopropanol (IPA), methanol; (2) HPLC grade ammonium acetate; and (3) ACS grade sodium carbonate and sodium chloride. ACS reagent grade formic acid (96%) was purchased from Aldrich (St. Louis, MO), and OmniSolv methyl *t*-butyl ether (MtBE) was purchased from E.M. Science (Gibbstown, NJ). Human control urine was provided by in-house staff (MRL, West Point, PA). Water was purified by a Milli-Q ultrapure water system from Millipore (Bedford, MA).

For use in solubilizing the internal standard **F** in stock solutions, a 0.2% (w/v) solution of formic acid was prepared in water. A solution of IPA-MtBE (10:90 v/v) was prepared as the extraction solvent. A solution of sodium carbonate (1.0 M, pH 11.0) was prepared to adjust the pH of urine before extraction. A sodium chloride solution (0.1 M) was prepared for washing the organic layer. Human control urine containing BSA (BSA urine) was prepared by mixing a 35% BSA solution with

urine to obtain a final level of 0.75% BSA in urine (v/v) and stored at −20°C.

For the mobile phase a solution of ammonium acetate buffer (0.01 M, pH 3.0) was prepared and the pH was adjusted with formic acid. The mobile phase for the LC–MS–MS assay was prepared by combining 630 ml acetonitrile, 30 ml methanol and 340 ml ammonium acetate (0.01 M, pH 3.0). For the LC-UV assay, mobile phase was prepared by combining 450 ml acetonitrile and 550 ml ammonium acetate buffer (0.01 M, pH 3.0).

2.2. Sample preparation

The sample preparation procedure prior to detection was similar between the HPLC-UV and HPLC–MS–MS assays. Control BSA urine blanks, standards, QC samples or clinical samples were thawed to room temperature. Aliquots of 1.0 ml of these samples were mixed with the following: 100 μl of working IS solution, 100 μl of **A** working standard (or acetonitrile in the case of QCs and clinical samples), and 100 μl of sodium carbonate (1.0 M) to adjust the final pH to 10.

For extraction, 6 ml of IPA-MtBE (10:90 v/v) was added to each sample tube. Samples were mixed for 5 min on a rotator (Glas-Col, Terre Haute, IN), centrifuged at 2000 × *g* for 5 min and the aqueous layer frozen in a methanol-dry ice bath. The organic layer was then decanted into a clean test tube. The extract was washed with 1.0 ml of NaCl (0.1 M w/v), centrifuged, frozen, and the organic layer decanted into a clean test tube. The organic phase was evaporated under a stream of air at 50°C in a Turbo-Vap evaporator (Zymark, Hopkinton, MA). The samples were reconstituted in 500 μl of mobile phase.

2.3. Preparation of calibration standards and QC samples

Calibration standards were prepared over a range of 5–500 ng/ml for the LC–MS–MS assay and 0.02–5 μg/ml for the LC-UV assay.

For the LC–MS–MS method a 100 μg/ml stock solution of **A** was prepared in acetonitrile in a 10-ml volumetric flask. The internal standard stock solution was 10 μg/ml of **F** in 10 ml of acetonitrile with 100 μl of 0.2% formic acid added to improve solubility. Working standards of **A** in acetonitrile were prepared at 0.05, 0.1, 0.2, 0.5, 1.0, 2.5 and 5.0 μg/ml and stored in 15-ml polypropylene screw-cap culture tubes at −20°C and were diluted to prepare final BSA urine standards. A working IS solution of 0.2 μg/ml of **F** was prepared daily in acetonitrile. BSA urine standards of **A** were prepared daily by adding 100 μl of working standard plus 100 μl of 0.2 μg/ml IS to 1 ml of BSA urine to provide final concentrations from 5 to 500 ng/ml.

For the LC-UV assay, a 1.0 mg/ml solution of **A** and a 100 μg/ml solution of **F** (containing 100 μl of 0.2% formic acid in 10 ml total) were prepared in acetonitrile as stock solutions. Working standards of **A** from 0.2 to 100 μg/ml in acetonitrile were prepared and stored in the same way as the LC–MS–MS assay. Working IS in acetonitrile was prepared daily at 5.0 μg/ml. A volume of 100 μl of working standard and 100 μl of 5.0 μg/ml IS were added to 1.0 ml of BSA urine for daily calibration standards ranging from 0.02 to 5 μg/ml.

For QC samples, stock solutions of **A** at 1.0 mg/ml, 100 μg/ml and 10 μg/ml were prepared in acetonitrile from a separate weighing of **A**. For the LC–MS–MS assay, QC samples were prepared at 10, 20, 100 and 250 ng/ml and for the LC-UV assay, at 50 and 750 ng/ml. The QC samples were stored at −20°C.

2.4. HPLC Conditions for LC-UV and LC–MS–MS Assays

For the LC–MS–MS assay, the mobile phase was composed of acetonitrile–methanol–ammonium acetate buffer (5 mM, pH 3.0) (63:3:34 v/v). The HPLC column was a BDS Hypersil C8, 3 μm, 50 × 4.6 mm column from Keystone Scientific (Bellefonte, PA). The injection volume of reconstituted sample was 40 μl. With a flow rate of 0.6 ml/min, the retention time was 1.8 min for the IS and 2.1 min for **A**.

An identical column was used for the LC-UV assay but the mobile phase was composed of acetonitrile–ammonium acetate buffer (0.01 M, pH 3.0) (45:55 v/v). The injection volume was 50

μl of reconstituted sample. Retention times were 2.9 min for the IS and 4.3 min for **A** with a flow rate of 1.0 ml/min.

2.5. Instrumentation for LC–MS–MS and LC-UV assays

Chromatography was performed on a Hewlett-Packard Series 1050 HPLC system (Hewlett-Packard Company, Wilmington, DE) for the LC–MS–MS method. The HPLC was coupled to a Sciex API 300 triple quadrupole mass spectrometer with a Sciex APCI interface (Sciex, Toronto, Canada) used in the positive-ion mode. For the UV assay, a Waters 600S Controller, 717 Plus Autosampler and 616 gradient HPLC pump (Waters Corporation, Milford, MA) were connected to an ABI Spectroflow 783 UV detector (ABI, Foster City, CA) that was set at 262 nm.

For the LC–MS–MS assay, the APCI interface was heated to 450°C and the nebulizing gas flow (N_2) was approximately 500 ml/min. The collision gas was N_2 ($CGT = 1.77 \times 10^{15}$ molecules/cm^2). Multiple reaction monitoring (MRM) was done in positive ion mode. Parent ions, m/z 592 → 439 for **A** and m/z 578 → 439 for **F** were monitored using a 250 ms dwell time. To minimize cross-talk on the API 300, dummy ions were placed between **A** and **F** during an MS/MS scan (m/z 700 → 300; 60 ms dwell). Peak area ratios for calibration curves and quantitation of unknowns were calculated using MacQuan software (Sciex, Toronto, Canada).

2.6. Method validation for LC-UV and LC-MS-MS assays

Intraday precision and accuracy were determined by analyzing replicate calibration curves ($n = 5$). Precision was calculated by determining the variability of the peak area ratio at each concentration over the standard range using MRM or UV detection. To calculate accuracy, measured concentrations for each standard were compared with nominal concentrations. The measured concentrations were generated from weighted least squares linear regression of five replicate calibration curves (regression of $n = 40$ points). For LC–MS–MS, a weighting factor of $1/x$ was used for regression calculations, where x = nominal concentration. The weighting factor for the UV assay was $1/y$, where y = peak area ratio. Freeze–thaw stability was evaluated for **A** at 10, 20, 100 and 250 ng/ml. QC samples were assayed before and after freezing at −20°C with three cycles of freezing and thawing and several days storage at −20°C between each thaw.

Frozen BSA urine QC samples at low, medium and high concentrations were prepared months before samples were collected at the clinical site, and stored at −20°C to determine interday assay variability and freezer stability. QC samples were thawed and analyzed daily in duplicate with a standard curve and clinical samples. Acceptance of sample concentration data was based on the QC results (< 20% different from nominal).

2.7. Extraction recovery

Liquid–liquid extraction efficiency for the LC–MS–MS assay was calculated by comparing peak areas of standard replicates at concentrations of 5, 10, 50, 250, and 500 ng/ml BSA urine with areas of neat standards in solvent.

2.8. LC–MS–MS procedure to determine qualitative metabolism of *A*

A qualitative LC–MS–MS assay was developed for alcohol, ketone and free amine analogs of **A** in BSA urine to investigate human metabolism.

Structures of analogs available for qualitative assessment of suspected oxidized metabolites **B**, **C,** and **D** and a free amine **E** are shown in Fig. 1. Analog **B** was used in place of **C** for qualitative analysis of suspected metabolites in urine because analogs **B** and **C** have similar mass spectral characteristics and response.

The urine LC–MS–MS metabolite assay involved the use of a BDS Hypersil C8 column (150 × 4.6 mm) and monitoring of additional parent → fragment ion pairs. For analogs **B**, **D** and **E** the ion pairs are m/z 608 → 439, 606 → 439 and 471 → 301, respectively. For LC–MS–MS on the Sciex API 365, cross-talk between the channels

arising from monitoring the same fragment masses was avoided by placing dummy ion transitions between each parent → fragment ion: m/z 700 → 300 for 60 ms, to clear the collision cell before the next transition.

2.9. Clinical study

In a Phase I, double-blind, placebo-controlled, rising, single-dose study, 36 subjects received single oral doses of 10, 25, 50, 125, 500 and 1000 mg of **A**. Urinary excretion (U_e) of parent compound was estimated as percent of dose.

In selected treatment periods, all urine voided during a collection interval was combined. Commercial 35% BSA solution was added to the urine collection container immediately following the void to an approximate concentration of 0.75% BSA in urine (2.14 ml of 35% BSA solution to 100 ml of urine). Clinical BSA urine samples were then covered with aluminum foil and stored at − 20°C for shipping.

3. Results

3.1. HPLC-UV assay

The HPLC-UV assay for **A** in 1 ml of BSA urine was validated for intraday precision and accuracy, and specificity over a range of 0.02–5 μg/ml in five separate lots of BSA urine (Table 1). The lower limit of quantitation (LLOQ) was 20 ng/ml.

The UV assay was free from endogenous interferences and was validated with acceptable precision and accuracy statistics. However, prior to the collection of clinical urine samples it was determined that the UV assay was not specific for **A**. When metabolite analogs of **A** (**B–E)** were chromatographed, **E** co-eluted with **A.** This occurred despite chromatographic separation of **A** from its oxidized analogs (**B–D**) (Fig. 2). Co-elution of A and E occurred despite attempts to resolve them using C18 and C8 HPLC columns (Hypersil, Spherisorb, Waters YMC, Zorbax) with mobile phases composed of acetonitrile and one of the following aqueous components: ammonium formate, ammonium acetate, both ammonium formate and acetate, and TFA.

3.2. Mass spectral characteristics of *A* and *F*

Parent $(MH)^+$ ions for drug and IS are shown in Fig. 3 and are m/z 592 and 578, respectively, for **A** and internal standard. Both **A** and internal standard had an intense product ion at 439 with other fragments at m/z 421 and 301. The major fragment, m/z 439, in **A** and **F** was from cleavage of the tetrazolone moiety. Fragment ions at m/z 421 and 301 are from loss of water and 2-hydroxypyridinylethylamine, respectively, from fragment m/z 439. Precursor/product ions chosen for MRM of **A** and IS were m/z 592 → 439 and m/z 578 → 439.

Table 1
Intraday accuracy and precision for the determination of **A** in human control urine containing 0.75% BSA: UV assay

Nominal conc. (ng/ml)	Mean calculated conc. (ng/ml)	Mean accuracy (calculated/nominal,%)[a]	Precision (CV,%)[b]
20	21.6	107	9.89
50	48.0	96.0	3.61
100	96.6	96.6	3.46
250	23.9	96.0	3.34
1000	1016	102	0.52
2500	2544	102	1.75
5000	5138	103	3.41

[a] Mean accuracy and precision calculated from least squares linear regression $1/y$ weighting of $n = 35$ points; five replicates per point with each replicate from a separate source of urine (Intercept = −0.036, Slope = 1.895, $R = 0.999$). The range of standards differs between the UV and LC–MS–MS assay due to the nature of detection, UV is less sensitive than LC–MS–MS.

[b] Coefficients of variation (CVs) for standard replicates are based on peak area ratios of **A** to internal standard.

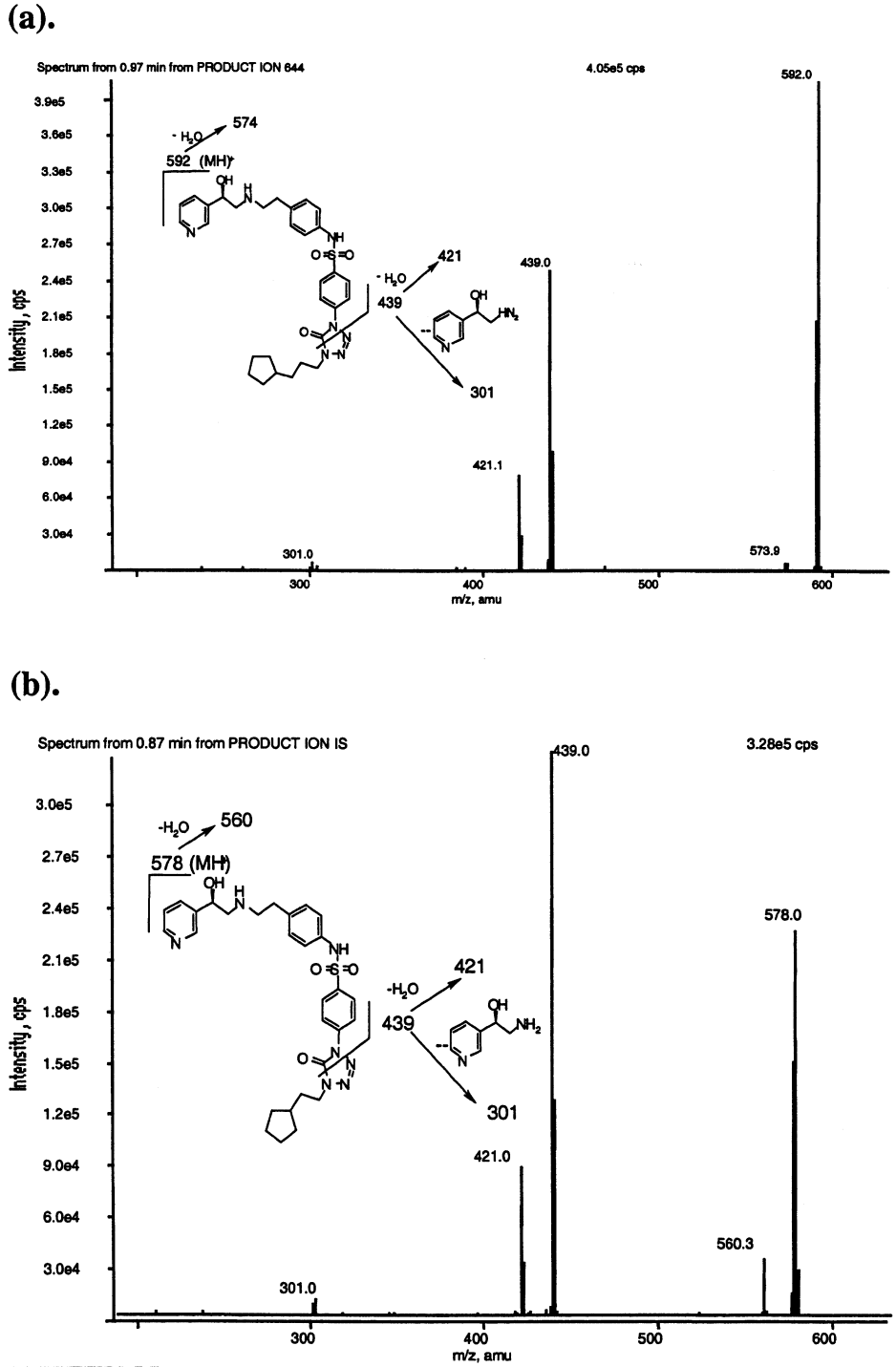

Fig. 3. Positive-ion APCI full-scan product ion of (a) B_3-agonist and (b) internal standard.

Table 2
Intraday accuracy and precision for the determination of **A** in human control urine containing 0.75% BSA: LC–MS–MS assay

Nominal conc. of standard (ng/ml)	Mean calculated conc. (ng/ml)	Mean accuracy (calculated/nominal,%)[a]	Precision (CV,%)[b]
5	5.74	115	3.94
10	10.5	105	2.06
20	18.9	94.5	2.13
50	45.6	91.2	1.38
100	94.7	94.7	2.07
250	237	94.6	1.60
500	523	105	1.87

[a] $n = 5$ at each different concentration, each replicate in a different lot of BSA urine to test specificity. Mean accuracy and precision calculated from least squares linear regression with $1/x$ weighting of $n = 35$ points (Intercept = −0.113, Slope = 0.045, $R = 0.998$). Intraday statistics for each of five standard curves regressed independently: Slope ranges from 0.044 to 0.046, intercept from −0.101 to −0.125, $R = 0.998$–0.999.

[b] CVs for standard replicates are based on peak area ratios of **A** to internal standard.

3.3. LC–MS–MS assay

The LC–MS–MS urine assay was specific for **A** using five different lots of BSA urine. Intraday accuracy and precision statistics over a calibration range of 5–500 ng/ml are listed in Table 2; interday precision for BSA urine QC samples at four separate concentrations are shown in Table 3. Intraday precision of peak area ratios ranged from 1.38 to 3.94%. Calculated concentrations ranged from 91.2 to 115% of nominal values. The variability in the QC samples after 3 months storage at −20°C at concentrations of 10, 20, 100 and 250 ng/ml were 2.0, 3.4, 4.0 and 4.9%, respectively. Individual calculated concentrations were within 90–111% of nominal concentrations. These changes were well within the precision error of the assay. The specific LC-MS-MS method replaced the non-specific UV method for clinical urine sample analysis. A representative chromatogram is shown in Fig. 4.

3.4. Comparison of LC-UV and LC-MS-MS assays

Clinical samples were analyzed for apparent **A** concentrations by the LC-UV method and the LC–MS–MS method. Levels of **A** were 2-fold higher (0–4 h urine collection) to 7-fold higher (12–24 h urine collection) when assayed by LC-UV as compared to LC–MS–MS (Table 4). The apparent concentrations of **A,** as measured by LC-UV, reflect levels of **E** and possibly other unidentified metabolites that co-elute with **A** (non oxidized metabolites). The levels of these metabolites increase over time. The total levels of **E** and **A** alone cannot account for the differences between assays.

3.5. Effects of BSA on recovery of *A* from human urine

During assay development for **A** in urine, levels of **A** were significantly reduced in QC samples (0.02 and 7.5 μg/ml) after storage at −20°C (Table 5). This problem was remedied by adding BSA to urine QC samples before storage. BSA has been used previously in our laboratories to

Table 3
Interday precision and accuracy for QC BSA urine samples[a] using LC–MS–MS assay

Parameter	QC sample concentration (ng/ml)			
Nominal	10	20	100	250
Mean[b]	10.8	20.1	95.6	245
S.D.	0.2	0.7	3.8	12
% CV	2.0	3.4	4.0	4.9

[a] Stored at −20°C for 3 months.

[b] $n = 5$ independent days of assay of QC samples, where two quality controls were analyzed per day. Mean and S.D. were calculated from $n = 10$ values.

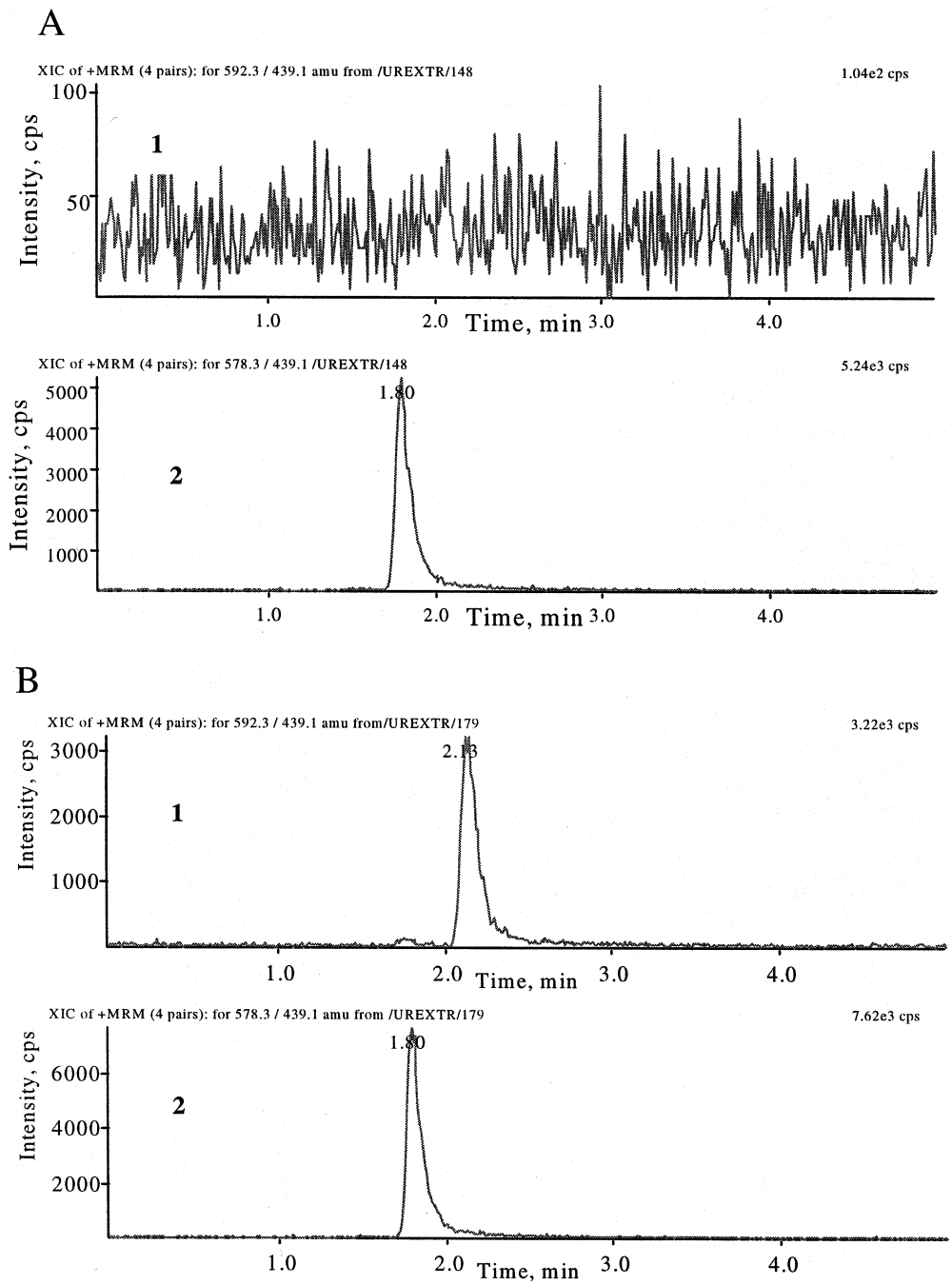

Fig. 4. Representative MRM chromatograms of extracts of human BSA urine samples (1 = drug channel; 2 = internal standard channel): (A) predose sample with internal standard, (B) postdose sample with drug (11 ng/ml) and internal standard.

Table 4
Ratio of LC-UV to LC–MS–MS results in BSA urine from subjects administered 1000 mg oral doses

	Ratio of concentration by LC–UV/LC–MS–MS			
Time, h	0–4	4–8	8–12	12–24
Subjects				
1	1.8	2.7	3.4	LLOQ[a]
2	LLOQ[a]	2.5	5.2	7.4
3	LLOQ[a]	LLOQ[a]	2.6	LLOQ[a]
4	2.0	4.0	4.9	6.8
5	1.5	3.4	LLOQ[a]	LLOQ[a]
Mean	1.8	3.2	4.0	7.1

[a] Less than detection limit for LC-UV.

prevent poor recovery of analyte from urine during storage and handling [6]. The optimal amount was 0.75% BSA in urine (Fig. 5). Clinical protocols were amended to require addition of BSA (2 ml of 35% BSA to each 100 ml of urine) to freshly collected subject urine samples prior to storage at −20°C at the clinical site.

The recovery of **A** from BSA urine, compared to neat standard, was measured by LC-MS-MS at five different concentrations over the standard curve (5, 10, 50, 250 and 500 ng/ml, $n = 5$ at each concentration) and varied from 81.6 ± 5.3 to $99.3 \pm 13.9\%$. The recovery of internal standard was 90.3% ($n = 30$).

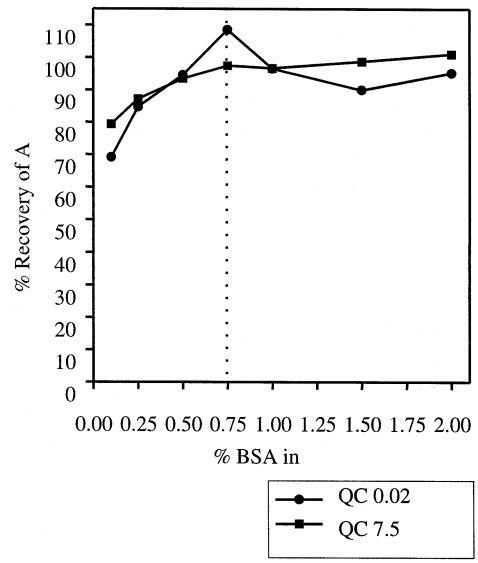

Fig. 5. Optimal BSA content for **A** in urine QCs.

Table 5
Effects of bovine serum albumin (BSA) on recovery of **A** from human urine

	Concentration of A[a] (μg/ml)							
	QC 0.02				QC 7.5			
	Fresh		Frozen[b]		Fresh		Frozen	
	No BSA	0.75% BSA	No BSA	0.75% BSA	No BSA	0.75% BSA	No BSA	0.75% BSA
	0.018	0.021	<LLOQ[c]	0.018	6.52	7.61	3.78	6.99
	0.020	0.021	<LLOQ	0.02	6.70	7.56	4.19	6.97
	0.016	0.021	<LLOQ	0.016	6.53	7.48	4.16	7.00
	0.016	0.022	<LLOQ	0.017	6.56	7.52	4.14	6.90
	0.021	0.026	<LLOQ	0.021	6.60	7.41	4.14	6.91
Mean	0.018	0.022		0.018	6.58	7.52	4.09	6.95
S.D.	0.0023	0.0023		0.0022	0.073	0.077	0.170	0.047
% CV	12.7	10.3		12.0	1.1	1.0	4.2	0.70
% Recovery	90.7	111.5		91.5	87.7	100	54.5	92.7

[a] Determined using UV method.
[b] −20°C.
[c] <LLOQ; limit of quantitation of HPLC-UV assay = 0.02 μg/ml.

3.6. Mass spectrometer conditions for investigation of human metabolism of A

In order to study the metabolism of **A**, a qualitative LC-MS-MS assay was developed to measure **A** and suspected metabolites. The common fragment ion m/z 439 was used for the detection of hydroxylated analog **B** and the ketone **D**; for the amine **E**, the most intense fragment ion m/z 301 was monitored. The same state file conditions were used to monitor **B**, **D** and **E** on the LC–MS–MS. These compounds were analyzed at μg/ml concentrations to evaluate for cross-talk in the collision cell. Drug and internal standard were free from cross-talk as shown in extracted ion chromatographic channels (Fig. 6). Although **B** was used in place of **C**, compound **C** is included here to demonstrate selectivity of the fast analytical HPLC column for the oxidized analogs of **A**. Extracted-ion chromatograms of predose and post dose BSA urine samples show no cross-talk or endogenous interferences between **A**, **F**, and analogs **B**, **D** and **E** despite similar fragmentation patterns (Fig. 7).

3.7. Metabolites found in urine determined by LC–MS–MS

Alcohol, ketone and free amine metabolites were found in urine samples (Fig. 7). The levels of oxidized metabolites (alcohol and ketone) were substantially higher than **A,** while concentrations of **E** were similar to **A** (Fig. 8).

Interday precision for the metabolite assay was calculated by comparing ratios of drug or metabolite to internal standard from 5 to 500 ng/ml over 5 separate days of analysis. The interday variability, expressed as% CV, at concentrations of 5, 10, 20, 50, 100, 200, 500 ng/ml ($n = 5$) ranged from 1.3 to 6.2% for **A**, 8.9 to 17.6% for **B**, 6.7 to 14.8% for **D** and 4.2 to 11.8% for **E**. Calculated concentrations were within 90–110% of nominal concentrations.

The recovery of **A** and analogs at three different concentrations (5, 50, and 200 ng/ml, $n = 3$) from BSA urine measured by LC–MS–MS varied from $89.1 \pm 11.4\%$ for **A**, $70.0 \pm 5.6\%$ for **B**, $73.5 \pm 5.2\%$ for **D** to $96.1 \pm 13.8\%$ for **E**.

4. Discussion

The LC–MS–MS method described for **A** in BSA urine was specific and sensitive to 5 ng/ml, and successfully applied to samples from subjects receiving single oral doses from 50 to 1000 mg of **A** to support clinical trials. The pharmacokinetic data suggests very little of **A** ($< 0.006\%$ of the dose) is excreted in the urine.

Acquiring and evaluating the alcohol, ketone and amine analogs of **A** before the clinical study started alerted us to the lack of specificity of the UV assay for **A** and allowed qualitative assessment of the metabolism of **A** for drug discovery efforts. This work emphasizes that potential metabolites should be obtained and chromatographed as early as possible in the development of a new method. Also crucial for accurate assay of **A** was identifying poor recovery of **A** in frozen urine QC samples before clinical urine samples were collected. The clinical protocol was amended to include addition of BSA to freshly voided urine before freezing at the clinical site allowing accurate estimation of **A** in urine samples.

Pharmacologically active oxidized metabolites and one inactive free amine metabolite were discovered in samples from a Phase I study (using the available analogs **B**, **D** and **E** as reference standards). Extracted ion chromatograms shown in Fig. 6 show **A** was metabolized into oxidized metabolites and a free amine metabolite**.** Levels of oxidized metabolites were substantially greater than concentrations of **A,** while levels of the amine **E** were similar to **A** (Fig. 8). The percent urinary excretion of parent drug, ketone, alcohol and free amine metabolite, on average, was less than 0.1% of dose (data not shown).

The location of hydroxylation cannot be determined from these experiments so hydroxylated analogs and metabolites may not be identical. Urine chromatograms suggest that there may be more than one isomer of ketone metabolite found in humans (Fig. 7). Since the mass spectral response in terms of sensitivity is nearly identical for **A** and its oxygenated analogs, we felt justified in making qualitative assessments of oxidized metabolite levels in urine.

The free amine metabolite found in urine from

humans dosed with **A** has identical chromatographic and mass spectral characteristics to synthetic analog **E**; therefore, the assessments for the amine are more quantitative than they are for the oxidized metabolites. As a result of metabolism work done in this area here and with other spe-

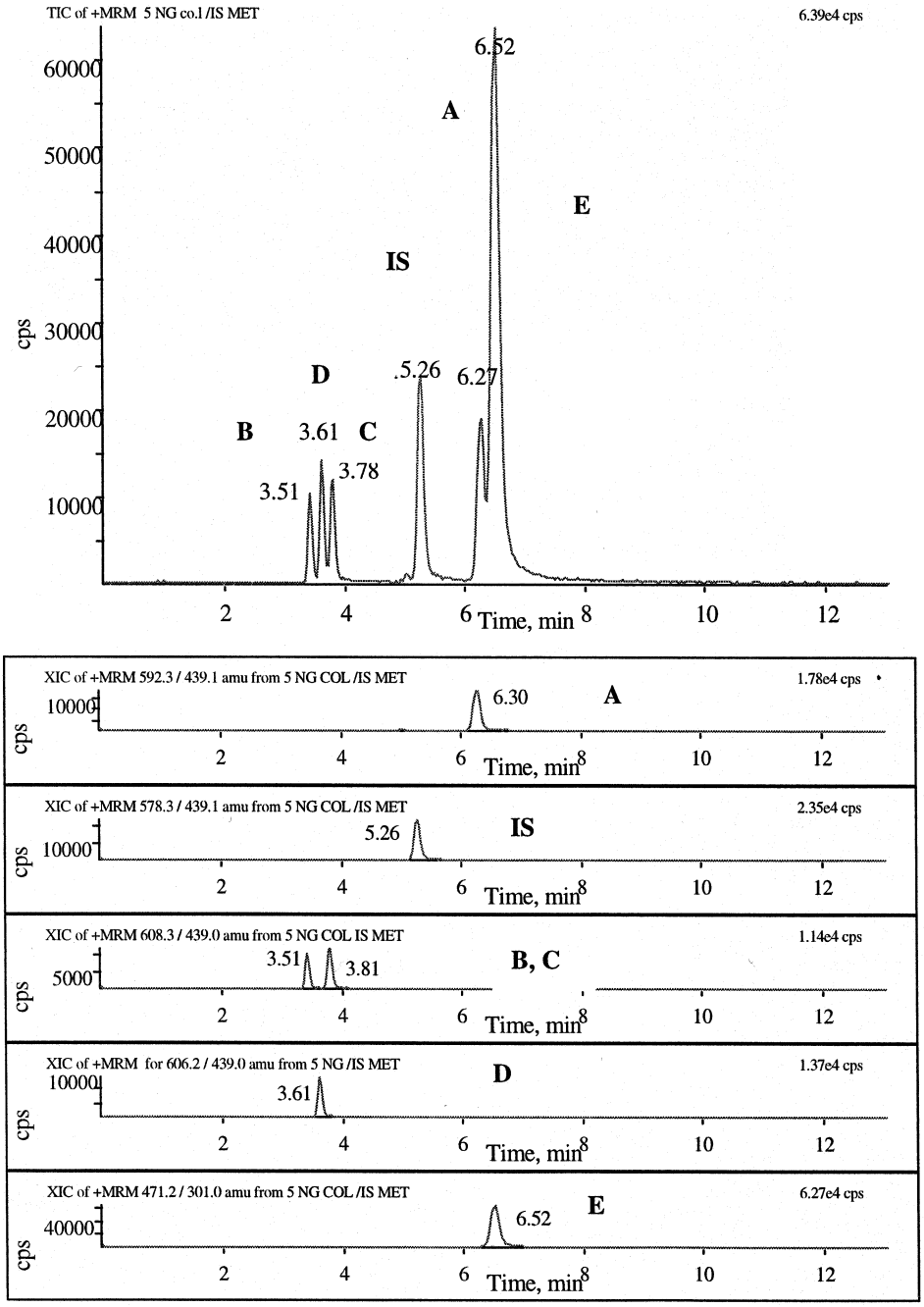

Fig. 6. Total and extracted ion current chromatograms for neat solutions of **A**–**E** and IS.

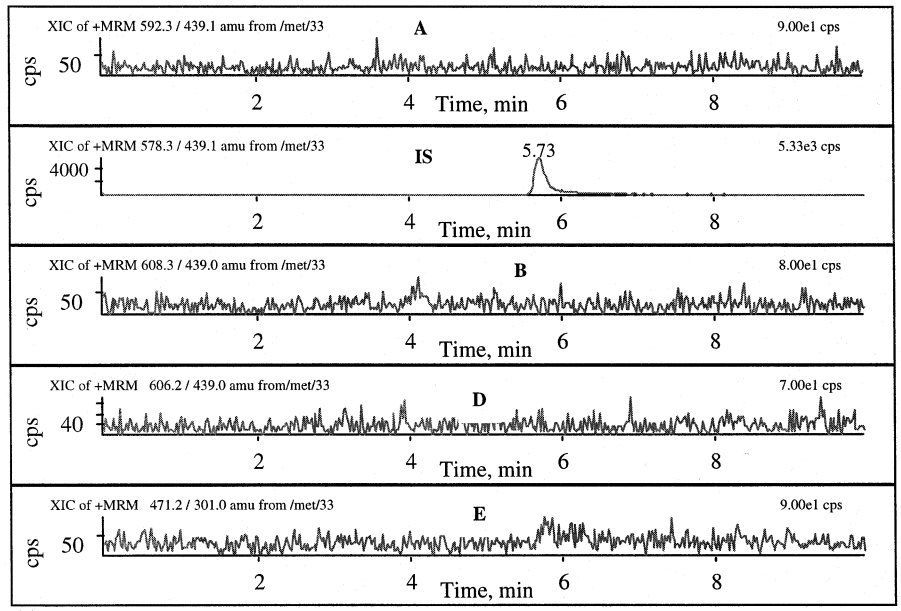

Fig. 7. (A) Representative MRM extracted ion chromatograms of a predose BSA urine sample from a patient (with internal standard **F** added). (B) Representative MRM extracted ion chromatograms of a BSA urine postdose sample containing drug (**A**), alcohol, ketone, amine suspected metabolites (**B**, **D**, **E**) and internal standard (**F**).

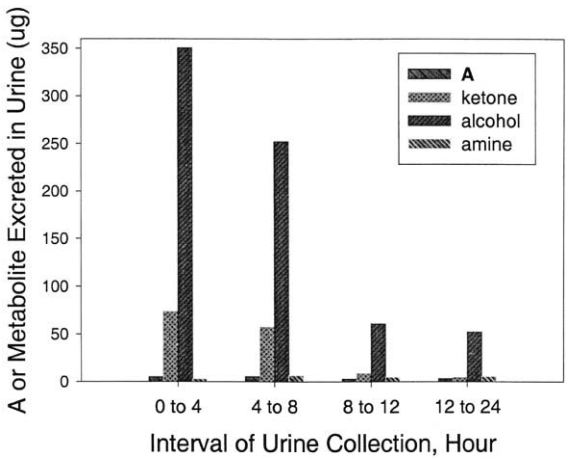

Fig. 8. Mean ($N+4$) qualitative urine profiles of **A** and suspected metabolites following 250 mg oral dose of **A**.

cies, a newer generation of compounds have been designed to avoid metabolism to hydroxylated species and free amine.

5. Conclusion

A specific LC–MS–MS assay for **A** in urine was developed to support Phase I studies. If the synthetic analogs of the suspected metabolites had not been available during development of the LC-UV assay, interference from the free amine metabolite may not have been detected, despite a very selective chromatographic system. Also, early discovery of adsorption problems to plastic containers allowed the clinical protocol to be amended for addition of BSA to freshly voided urine at the clinical site. Qualitative assessment of metabolism of **A** in human urine was useful for assay development to support Phase I clinical studies and in elucidating the extensive metabolism of this class of compounds during drug discovery. Subsequent efforts to develop β_3 adrenergic receptor agonists were designed to reduce metabolism in these regions of the molecule.

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