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Effect of the sample matrix on the determination of indinavir in human urine by HPLC with turbo ion spray tandem mass spectrometric detection[1]

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Abstract

The HPLC/tandem mass spectrometric (LC/MS/MS) behavior of indinavir, an HIV protease inhibitor, in human urine is presented as an example of a case where endogenous matrix components were found to interfere with the ionization of the target analyte. The MS/MS system used for these experiments was equipped with a turbo ion spray LC interface. Results from two sample preparation procedures (direct dilution of urine vs urine extraction) and two chromatographic systems (low vs. high capacity factor (k')) for the analytes were compared. Additionally, the precision of the analysis that was achieved while using a stable isotope labeled internal standard is contrasted with the results obtained using an analog of indinavir as internal standard. The results obtained indicated that during development and validation of LC/MS/MS based assays the potential effect of co-eluting 'unseen' endogenous species should be evaluated to ensure that sample preparation and chromatography is adequate to overcome the matrix effect problems.

Keywords: Indinavir; Matrix effect; Liquid chromatography–tandem mass spectrometry; Turbo ion spray

1. Introduction

HPLC with tandem mass spectrometric detection (LC/MS/MS) is an analytical technique that is widely used in the pharmaceutical industry for the determination of drugs in biological fluids [1–3]. A number of examples from our laboratories demonstrating the applicability of this method have been described [4–11]. It is generally assumed that the highly specific nature of LC/MS/MS permits the use of short chromatographic analysis times and minimal sample clean-up procedures. Thus, high sample throughout is often emphasized as a major advantage of this technique. Large amounts of endogenous species may potentially co-elute with the target analyte when

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minimal sample preparation procedures are combined with short analysis times. While these co-eluting species are invisible to the detector when only product ions of the analyte are monitored, they may, however, significantly affect the efficiency and reproducibility of the ionization process that takes place in the LC interface. Results describing the effect of co-eluting species on ionization efficiency using an ion-spray interface have recently been reported [12].

Indinavir is a potent and specific in vitro inhibitor of the human immunodeficiency virus Type 1 (HIV-1) encoded protease [13,14]. The compound has recently been approved for marketing in the US and other countries for the treatment of HIV infection under the name Crixivan®. In this report, the turbo ion-spray ionization behavior of indinavir in human urine samples is presented as an example of a case where endogenous matrix components were found to interfere with the ionization of the target analyte. The characterization of this effect and methods to overcome these interferences are described.

2. Experimental

2.1. Materials

Unlabelled indinavir (d_0-indinavir, monohydrate free base) (**I**, Fig. 1) and L-754394 (**III**, Fig. 1) were obtained from the Chemical Data Department of Merck Research Laboratories, Rahway, NJ. Deuterated indinavir (d_6-indinavir, monohydrate free base) (**II**, Fig. 1) was prepared by M. Braun of the Department of Drug Metabolism, Merck Research Laboratories. The chemical purities of all three compounds were over 99%.

Acetonitrile and water (Omnisolv HPLC grade) were from EM science (Gibbstown, NJ). Formic acid (A.C.S. reagent) and ammonium acetate (99.9%) were purchased from Aldrich (Milwaukee, WI). Other reagents were A.C.S. grade or better and were used as received.

Control human urine was obtained from volunteer staff members of the Department of Drug Metabolism of Merck Research Laboratories.

2.2. Instrumentation

The LC/MS/MS system consisted of a Perkin–Elmer (Norwalk, CT) model 250 pump, a Waters (Milford, MA) WISP 715 autosampler, and an API III plus triple quadrupole tandem mass spectrometer equipped with a turbo ion spray (TISP) interface (PE-Sciex, Thornhill, Canada).

2.3. Chromatographic conditions

Mobile phase for chromatographic system **A** consisted of 40/60 (v/v%) acetonitrile/7 mM ammonium acetate while the mobile phase for chromatographic system **B** was composed of a 30/70 (v/v%) solution of acetonitrile/7 mM ammonium acetate. Both mobile phases were adjusted to pH 4.9 with formic acid and were filtered through a nylon membrane (0.20 μm) prior to use. A Keystone Scientific (Bellafonte, PA) BDS Hypersil C8 (50x2.0 mm, particle size = 3 μm) column was used with both mobile phases. A flow rate of 0.2 ml min^{-1} was utilized for systems **A** and **B**. The sample injection volume was 6 μl and the run times were 6 and 12 min, when using systems **A** and **B**, respectively.

2.4. Mass spectrometric conditions

The mass spectrometer was connected to the HPLC system via a turbo ion spray interface (PE-Sciex) consisting of an articulated ion spray inlet [15] and a heated turbo probe. The turbo probe was operated at 500°C with an auxiliary gas (nitrogen) flow of 7 l min^{-1}. Nebulizer (nitrogen) pressure was set at 60 p.s.i. The mass spectrometer was operated in the positive-ion mode. Ions were generated in the ion-spray interface via an ion evaporation mechanism [16,17]. The interface sprayer was maintained at +4 kV, while the sampling orifice was set at +60 V. The first quadrupole, Q1, was set to monitor the protonated molecules $(M+H)^+$ at m/z 614, 620, and 654 for **I**, **II**, and **III**, respectively with collision-induced fragmentation at Q2 (collision gas argon, 240×10^{12} atoms cm^{-2}). Product ions were moni-

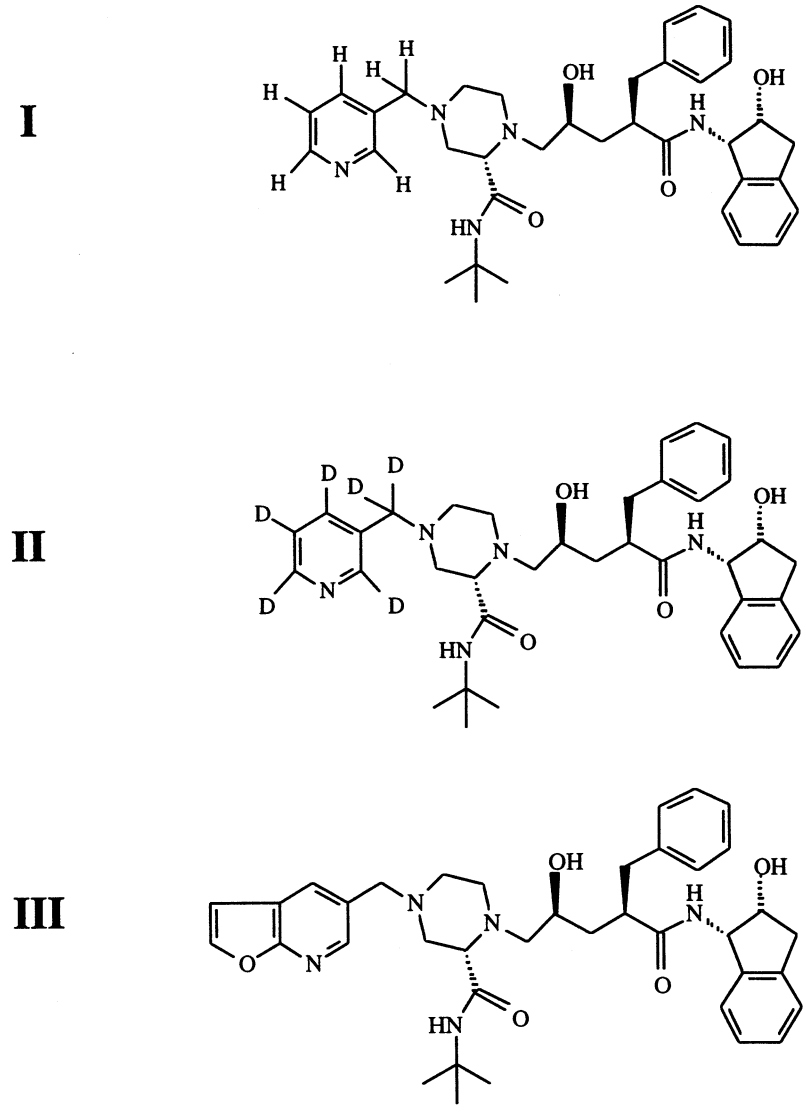

Fig. 1. Structures of **I** (d_0-indinavir), **II** (d_6-indinavir) and **III** (L-754394).

tored via Q3 at m/z 465, 471, and 505 for the respective analytes. The decision to use these particular product ions to monitor **I**, **II**, and **III** was based on the results of our previously described studies [18]. Q1 and Q3 were operated at unit mass resolution [1]. The electron multiplier setting was −4.1 kV and detector electronics were set to counts of 1. The dwell time was 500 ms with a 5 ms pause between scans. In order to eliminate 'cross-talk' between channels, Q2 settling with a park mass of 20 was used.

2.5. Preparation of standards

Stock solutions (10 μg ml^{-1}) of **I**, **II** and **III** were prepared by weighing amounts of the reference materials equivalent to 1.00 mg of anhydrous free base (1.03 mg for **I** and **II**, 1.00 mg for **III**) into 100 ml volumetric flasks, dissolving the compounds in 50 ml of acetonitrile, and filling each of the flasks to volume with water. The stock solution of **I** was further diluted with 50/50 (v/v%) acetonitrile/water to a final concentration of 4 μg ml^{-1}.

2.6. Sample preparation

2.6.1. Dilution procedure

A 1 ml aliquot of human control urine was spiked with 50 μl of the 4 μg ml^{-1} solution of **I**, and 25 μl of the 10 μg ml^{-1} solutions of **II** and **III** followed by an addition of 650 μl of acetonitrile. A 6 μl aliquot of the resulting solution was injected into the HPLC system for analysis.

2.6.2. Liquid–liquid extraction procedure

To a 1 ml aliquot of urine spiked with **I**, **II**, and **III**, as described above, was added 1 ml of 0.1 M pH 9.5 borate buffer. The resulting solution was extracted with 8 ml of methyl-*t*-butyl ether. Following the extraction, the organic layer was removed and evaporated to dryness under a stream of nitrogen. The resulting residue was reconstituted in 1.75 ml of the mobile phase for either chromatographic system **A** or **B**. A 6 μl aliquot of the resulting solution was injected into the HPLC systems for analysis.

2.7. Data acquisition and analysis

Data acquisition and analysis were performed with RAD software (PE-Sciex). MacQuan software (PE-Sciex) was used to determine the areas of the analyte peaks and calculate peak area ratios. Peak capacity factors were calculated based on a column void volume of 138 μl, as specified by the column manufacturer.

3. Results

3.1. Chromatographic separation

In order to evaluate the effect of peak capacity factor (k') on instrumental response, two chromatographic systems were developed. Utilization of chromatographic system **A** resulted in the coelution of analytes **I** and **II** at a retention time of 1.8 min ($k' = 2.6$). Under these conditions, analyte **III** eluted at 2.6 min with k' of 4.2. Representative chromatograms obtained using system **A** are shown in Fig. 2. Under the conditions of chromatographic system **B**, analytes **I** and **II** were slightly separated with retention times of 4.8 and 4.7 min (k' of 8.6 and 8.4), respectively (Fig. 3). Analyte **III** was further retained and eluted at 9.1 min (k' of 17.2) (Fig. 3). Use of a mobile phase with decreased organic content necessitated an increase in run time from 6 to 12 min when the chromatographic system was switched from **A** to **B**.

3.2. Sample preparation/instrument response

Two sample preparation schemes, direct injection of diluted urine and a urine extract obtained as a result of a liquid–liquid extraction procedure, were evaluated with respect to the absolute instrument response (peak area) produced by each analyte in each of five different pools of human urine. Samples containing **I**, **II**, and **III** at concentrations of 200, 250 and 250 ng ml^{-1}, respectively, were processed using both procedures and injected into chromatographic systems **A** and **B**. The absolute peak area responses for each analyte obtained under the different sample preparation and chromatographic conditions are shown in Table 1. Samples of control urine prepared using the two sample preparation schemes showed no interfering peaks in either chromatographic system.

3.3. Recovery of liquid–liquid extraction procedure

To determine the absolute recovery of the liquid–liquid extraction procedure, control samples from each of the urine pools were prepared using the liquid–liquid extraction procedure. Following phase separation and isolation of the organic layer, the methyl-*t*-butyl ether was spiked with the amount of each of the analytes that was initially present in the extracted urine samples described above. The spiked organic phase was then evaporated to dryness, reconstituted, and injected into each of the chromatographic systems. A comparison of the peak area of the samples spiked pre and post extraction indicated that the recovery of the liquid–liquid extraction procedure was greater than 90% for each of the analytes in each of the urine pools.

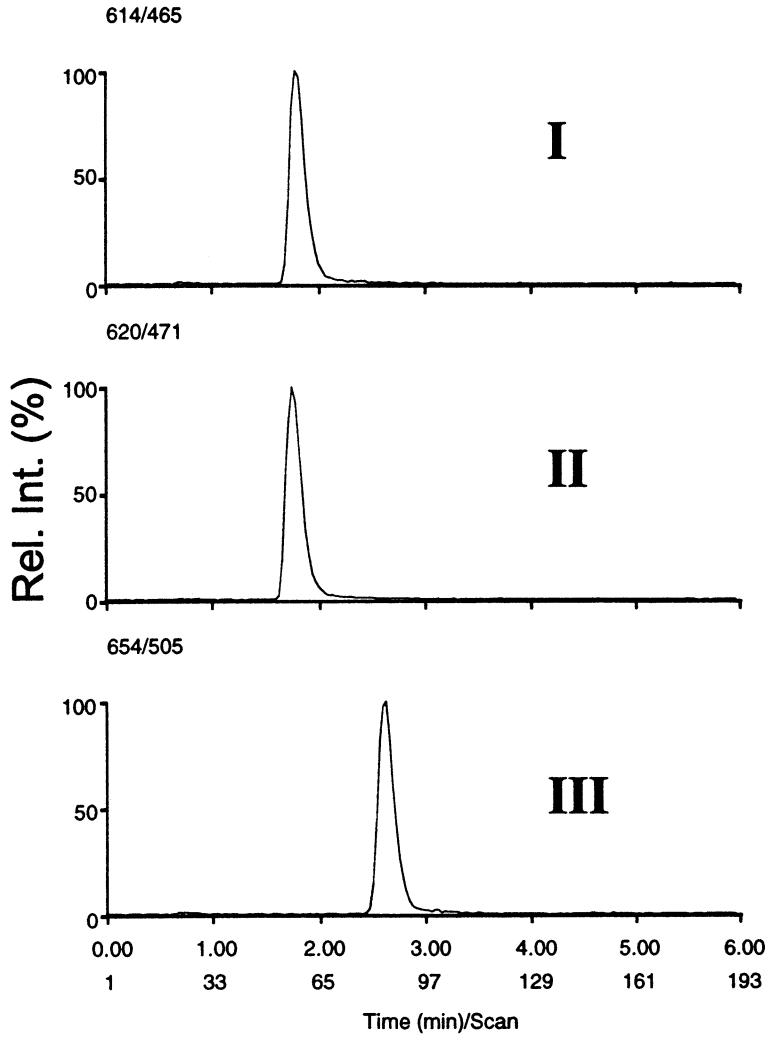

Fig. 2. Representative chromatograms of **I**, **II**, and **III** under the conditions of chromatographic system **A**.

3.4. Effect of matrix components on instrument response

For samples processed using the dilution procedure, the change in the efficiency of ionization for each analyte that could be attributable to the sample matrix was calculated by dividing the peak area of each of the analytes in the sample by that of an equivalent amount of each analyte injected into the system in mobile phase, converting this figure to a percentage and subtracting 100 from that amount. In the case of samples processed using the liquid–liquid extraction procedure, the matrix effect on ionization was calculated based on a comparison of the peak area of the analytes in the samples spiked post extraction to that of each analyte injected into the systems in mobile phase. The results of these calculations are presented in Table 2.

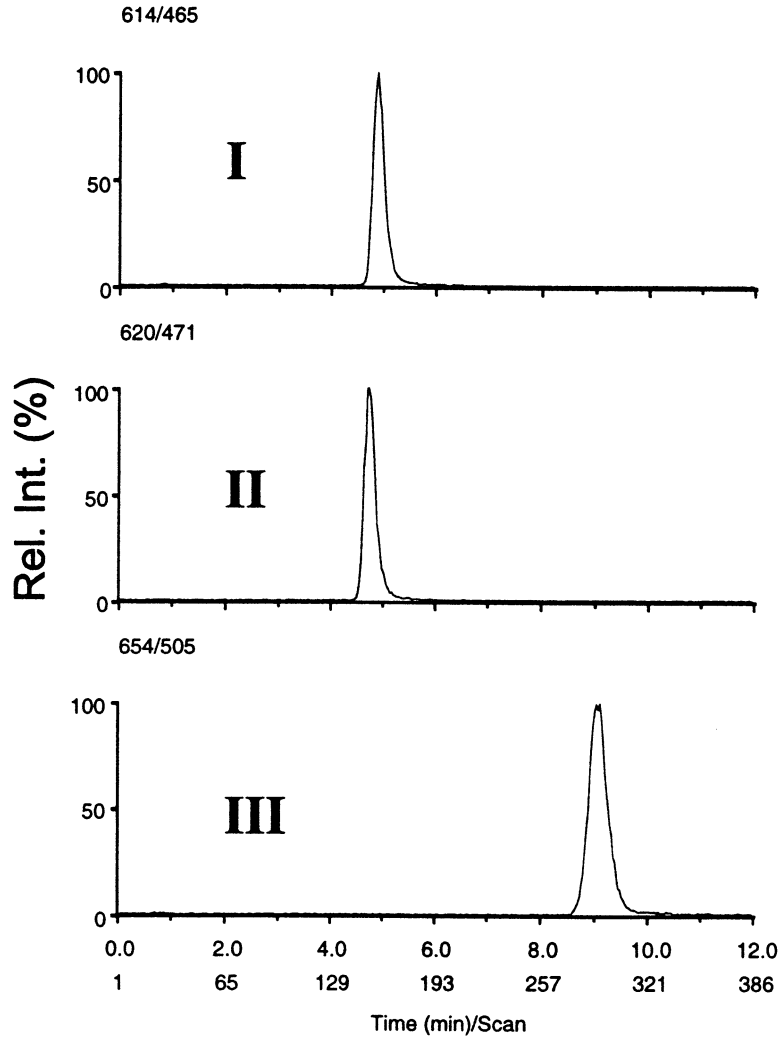

Fig. 3. Representative chromatograms of **I**, **II**, and **III** under the conditions of chromatographic system.

*3.5. Evaluation of analytes **II** and **III** as an internal standard for the determination of **I***

Either analyte **II** or **III** could potentially serve as an internal standard for the analysis of **I** in urine. In order to determine whether the use of either compound would serve to improve the precision of the instrument response, the ratios of the peak area responses of **I**/**II** and **I**/**III** were determined and the coefficient of variation across urine pools was calculated for each of the two sample preparation procedures utilized with each of the chromatography systems. The results are shown in Table 3. The corresponding peak area ratios and the coefficient of variation across urine pools are also shown in Table 3 for those samples that were spiked post liquid–liquid extraction.

4. Discussion

4.1. Sample matrix effects

We have previously described an LC/MS/MS assay for the simultaneous determination of **I** and **II** in plasma using **III** as internal standard [18].

Table 1
Effect of chromatographic system and sample preparation method on instrument response[a]

Analyte	Urine Pool	HPLC System **A** sample prep. method		HPLC System **B** sample prep. method	
		Dilution	L–L Extract[b]	Dilution	L–L Extract[b]
I	1	46 046	112 599	106 664	113 983
	2	61 110	110 903	109 615	104 375
	3	50 114	114 533	104 954	109 038
	4	69 946	111 884	107 679	115 265
	5	44 428	111 078	95 759	117 033
	Mean ($n=5$)	54 329	112 199	104 934	111 939
	C.V. (%)	20.0	1.3	5.1	4.6
II	1	50 098	119 983	112 329	119 683
	2	67 329	118 175	118 549	110 989
	3	55 642	121 570	113 882	115 855
	4	76 589	117 342	116 244	123 697
	5	49 725	116 647	99 748	124 400
	Mean ($n=5$)	59 876	118 743	112 150	118 907
	C.V. (%)	19.6	1.7	6.5	4.7
III	1	29 588	64 616	60 310	69 720
	2	39 231	64 991	62 758	60 627
	3	32 910	68 344	61 012	68 169
	4	40 189	64 829	64 390	72 399
	5	32 314	70 298	61 319	71 552
	Mean ($n=5$)	34 846	66 615	61 958	68 493
	C.V. (%)	13.3	3.9	2.6	6.9

[a] Mean peak areas from duplicate sample analyses are shown for each urine pool.
[b] Liquid–liquid extraction.

Because of the desire to determine **I** and **II** at low concentrations, a sample preconcentration/purification step in the form of a liquid–liquid extraction was incorporated into the LC/MS/MS sample preparation scheme for the plasma assay. Following oral administration of indinavir to man, approximately 10–20% of the dose is excreted unchanged in urine [19]. Hence, concentrations of indinavir in human urine typically fall in the high nanogram to the low microgram per milliliter range following the administration of an 800 mg dose. In theory, the high indinavir concentrations in urine combined with the highly specific nature of tandem MS detection should allow an assay for indinavir in urine to be developed based on the direct injection of small volumes of urine samples into the LC/MS/MS system; sample pretreatment to eliminate matrix components and concentrate the sample should not be required. Therefore, our initial approach to developing an assay for indinavir in urine was based on dilution of the sample with acetonitrile so that the organic content of the sample was approximately equal or less than that of the mobile phase, followed by direct injection into the TISP LC/MS/MS system.

Injection of urine samples, spiked with **I**, **II**, and **III**, that were prepared from five different pools of control urine and diluted with acetonitrile prior to injection into the HPLC system used for the plasma assay (system **A**) indicated that there was a high degree of variability in instrument response across urine pools (Table 1). Additionally, under these conditions, it was observed that the responses of the analytes in the urine samples were less than those of standards of **I**, **II**, and **III** dissolved in mobile phase and injected into the system (Table 2). For several urine pools the response decrease was greater than 60% relative to the standards in mobile phase.

The source of the decreased response was be-

Table 2
Effect of matrix components on instrument response

Analyte	Urine pool	HPLC System **A** sample prep. method		HPLC System **B** sample prep. method	
		Dilution[a]	L–L extract[b]	Dilution[a]	L–L extract[b]
I	1	−60.4	−13.6	−8.2	−0.4
	2	−47.4	−8.1	−5.6	5.7
	3	−56.9	−2.3	−9.6	5.3
	4	−39.8	−1.1	−7.3	4.5
	5	−61.7	2.8	−17.5	4.9
II	1	−59.5	−14.2	−9.3	1.8
	2	−45.6	−7.9	−4.3	6.5
	3	−55.1	−4.1	−8.0	7.6
	4	−38.2	−1.0	−6.1	6.9
	5	−59.8	3.0	−19.4	3.9
III	1	−48.1	−4.9	5.8	19.3
	2	−31.2	−1.6	10.1	22.8
	3	−42.3	8.8	7.0	25.9
	4	−29.5	13.6	13.0	23.2
	5	−43.3	20.3	7.6	25.7

[a] Difference (%) in peak area of samples prepared using the dilution procedure relative to that of standards in mobile phase directly injected into the LC/MS/MS system.
[b] Difference (%) in peak area of samples spiked following the liquid–liquid extraction procedure relative to that of standards in mobile phase directly injected into the LC/MS/MS system.

lieved to be the presence of co-eluting endogenous components from the urine matrix. Ionization within the TISP interface is based on an ion-evaporation mechanism [16,17] whereby charged droplets exiting the sprayer are converted, with the assistance of a perpendicular stream of heated nitrogen, to gas phase ions that can enter the mass spectrometer. Co-eluting species may increase or decrease the efficiency of either the charged droplet formation process or the droplet evaporation process. Additionally, gas phase proton transfer reactions between ions of co-eluting species may occur, which may effect the population of analyte ions that are available to enter the mass spectrometer. Each of these processes have been proposed to affect the ionization process in the conventional electrospray MS interface [12,20].

Two approaches were utilized to evaluate whether a decrease in the presence of co-eluting species would result in an increased instrumental response that was consistent between urine pools. The first approach involved extracting the samples prior to injection. The liquid–liquid extraction scheme, previously utilized for the plasma assay, was applied to the urine samples. The evaporated samples were reconstituted in 1.75 ml of mobile phase, so that their volume was equal to that of the samples that were diluted prior to injection. Injection of the extracted samples into HPLC system **A** resulted in a significant increase in instrumental response relative to the samples that were simply diluted prior to injection (Table 1). To separate the contribution attributable to recovery losses occurring during the extraction process from the effect matrix components had on response, the peak area of samples spiked with **I**, **II**, and **III** following the extraction procedure was compared with that of the standards injected in mobile phase (Table 2).

For analytes **I** and **II**, the largest degree of matrix suppression in the diluted samples was observed in urine pool 1. In this pool, the degree of signal suppression was reduced from approximately 60% in the diluted samples to about 14% in the extracted samples. Curiously, for analyte

Table 3
Influence of internal standard on precision as assessed by the coefficient of variation of peak area ratios[a]

Peak area ratio	Urine pool	HPLC System **A** sample prep. method			HPLC System **B** sample prep. method		
		Dilution	L–L extract.	Post ext. spike	Dilution	L–L extract.	Post ext. spike
I/II	1	0.919	0.938	0.945	0.949	0.953	0.918
	2	0.908	0.939	0.937	0.925	0.941	0.931
	3	0.901	0.943	0.956	0.922	0.944	0.918
	4	0.913	0.952	0.886	0.927	0.932	0.916
	5	0.893	0.953	0.936	0.962	0.942	0.946
	Mean (n = 5)	0.907	0.94 5	0.932	0.9 37	0.94 2	0.926
	C.V. (%)	1.2	0.7	2.9	1.9	0.8	1.4
I/III	1	1.556	1.742	1.854	1.768	1.634	1.705
	2	1.562	1.707	1.904	1.747	1.722	1.755
	3	1.523	1.676	1.831	1.721	1.601	1.705
	4	1.741	1.727	1.858	1.673	1.593	1.729
	5	1.374	1.581	1.742	1.561	1.636	1.703
	Mean (n = 5)	1.551	1.838	1.686	1.694	1.637	1.719
	C.V. (%)	8.4	3.8	3.2	4.9	3.1	1.3

[a] Mean values from the duplicate sample analyses are shown.

III, all diluted samples showed significant signal suppression, however, a signal enhancement was observed in several samples from pools spiked post extraction relative to the standard injected in mobile phase. Presumably, the co-eluting species in the spiked post-extraction samples act to increase the efficiency of ionization of **III** in these samples relative to that in the samples injected in mobile phase. Such behavior is not entirely unexpected in light of the complex nature of the ionization process.

In addition to increasing peak area, the extraction procedure also improved the reproducibility of the peak areas of the analytes between urine pools. For example, in the case of analyte **I**, the precision (%CV) of the peak areas for the samples prepared via dilution is approximately 20%, while for those spiked following extraction the precision was better than 7%. The improvement in precision is probably due to the fact that endogenous species, which are present in varying amounts in the different urine pools, are significantly reduced in the samples processed via the extraction procedure.

Increasing the capacity factors (k') of the analytes in the chromatography system used for analysis in order to attempt to chromatographically resolve them from endogenous components was evaluated as a second means of reducing matrix effects. Reducing the acetonitrile content of the mobile phase from 40% (system **A**) to 30% (system **B**) was found to approximately triple the retention times of the analytes. Urine samples prepared using the dilution procedure and analyzed using chromatographic system **B** showed a significantly higher response than those analyzed with system **A**, indicating that the chromatographic removal of endogenous matrix components led to a more efficient ionization of analytes in system **B** (Table 1). Use of system **B** was also found to reduce, by a factor of at least three, the peak area variability when the results, for all analytes, from diluted urine samples from the different pools were compared.

The combination of extraction with increased chromatography was found to only modestly effect the magnitude and the precision of the analyte peak areas in urines from different pools.

Thus, either sample extraction or increased chromatography was found to be sufficient to reduce the contribution of matrix components to assay variability.

4.2. Evaluation of internal standards

Internal standards are generally incorporated into an assay in order to improve precision. Thus, incorporation of a suitable internal standard could provide a further means of eliminating assay variability due to matrix effects in assays based on tandem MS detection.

The primary requirement for an internal standard in a bioanalytical procedure is that it mimics the behavior of the target analyte. The major source of imprecision in assays that utilize conventional detection methods, such as UV or fluorescence, is the variability that occurs during the sample preparation step. Hence, in order for an internal standard to improve the precision of UV or fluorescence assays its extraction efficiency must track with that of the analyte. Due to relatively long run times and high k' values of the analytes, endogenous species in UV or fluorescence assays generally do not influence the precision of detection of the analyte.

In the case of an LC/MS/MS assay for indinavir in urine, co-eluting species were found to influence the detection of the analyte. Therefore, if an internal standard is to improve the precision of the assay, it must mimic the behavior of the analyte both during the sample preparation procedure and during detection in the TISP MS/MS system.

Both **II**, a stable isotope analog of **I**, and **III**, a structural analog of **I**, were found to mimic **I** during the sample preparation procedures. In order to see whether matrix components influenced the ionization behavior of **II** and **III** in the same manner as **I**, the ratios of the instrumental response (peak area) of **I** to that of **II** and **I** to **III** were calculated for spiked samples prepared from each of the urine pools. Based on the results presented in Table 3, it is clear that the behavior of **II** in the MS most closely matched that of **I**. In the case of samples prepared using the dilution procedure and analyzed via HPLC system **A**, the coefficient of variation of the peak areas of **I** was 20.0% (Table 1) compared to a CV of 1.2% when the ratios of the responses of **I** to **II** were calculated (Table 3). Furthermore, the precision of the peak area ratios of **I** to **II** were found to be relatively constant regardless of which sample preparation or chromatography system was used.

The ionization of the structural analog, **III**, was found to only partially mimic that of **I**. The CV of the ratios of the responses of **I** to **III** was found to be 8.4% for those samples that were diluted and analyzed via HPLC system **A** (Table 3). Whereas additional sample preparation or increased chromatography did not alter the precision of the peak area ratios of **I** to **II**, utilization of either the liquid–liquid extraction or increased chromatography did improve the precision from 8.4 to 3.8 and 4.9%, respectively when peak area ratios of **I** to **III** were calculated. In addition, the **I**/**III** ratios were significantly different (Table 3) when diluted samples analyzed via system **A** were compared with extracted samples. These differences were practically eliminated when chromatography system **B** was utilized.

Therefore, when structural analogs such as **III** are utilized as internal standard, they may only partially compensate for variable ionization effects caused by matrix components when minimum sample preparation or little chromatographic separation is applied. An assay for **I** in urine using **III** as internal standard would require either sample preparation via liquid–liquid extraction or extended chromatography in order to maximize precision, improve accuracy and eliminate matrix effects on ionization, the degree of which may be different for the two analytes **I** and **III**.

5. Conclusions

Matrix components have been shown to affect the ionization behavior of **I**, **II**, and **III** in human urine samples analyzed using a tandem MS system equipped with a TISP. Although the co-eluting endogenous species from urine were not seen in chromatograms obtained by monitoring product ions in the selected ion monitoring mode,

their presence appeared to either suppress or enhance the ionization of analytes, resulting in increased variation in their MS/MS responses. Both sample clean-up through liquid–liquid extraction and more chromatographic separation was shown to improve the instrument response and reproducibility of ionization, thus potentially improving sensitivity and precision of quantitation. Use of a stable isotope labeled internal standard was found to improve the precision of the analyte response, however, if the use of a structural analog as internal standard is necessary, a careful assessment of matrix effects is required and removal of endogenous components via sample extraction or increased chromatography is needed if such an effect is observed.

The matrix effect problem addressed in this communication is most likely not unique to indinavir. Therefore, to ensure reliable quantitation of analytes in post-dose biological fluids and assure the integrity of pharmacokinetic data, evaluation of the potential effect of co-eluting 'unseen' species arising from the sample matrix must be adequately evaluated before an assay is utilized to support large scale clinical studies with biological fluid samples originating from a large number of subjects collected over a long period of time. The evaluation and elimination of matrix effects should constitute an integral part of the development and validation of assay methods based on LC/MS/MS detection.

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