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Comparison of *p*-fluoroketorolac and [$^{18}O_3$]ketorolac for use as internal standards for the determination of ketorolac by gas chromatography/mass spectrometry (GC/MS)

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Abstract

A chemical and a stable-isotope analog, *p*-fluoroketorolac and [$^{18}O_3$]ketorolac respectively, were directly compared for applicability as internal standards for the determination of ketorolac in plasma samples using gas chromatography/mass spectrometry (GC/MS) with selective-ion-monitoring detection, following derivatization to form the methyl esters. This comparison involved analyzing ketorolac calibration standards and spiked plasma samples that contained both internal standard candidates. The response for ketorolac and each internal standard was monitored simultaneously and electronically integrated peak heights were obtained. Thus, for each analysis performed, a response ratio was obtained for each internal standard relative to an identical ketorolac response. Linearity of response for ketorolac calibration standards and accuracy for spiked plasma sample analysis were compared using each internal standard. The use of [$^{18}O_3$]ketorolac as the internal standard provided superior accuracy data for the analysis of ketorolac in plasma samples.

Keywords: Stable isotope dilution mass spectrometry; Internal standard; Calibration; Linear regression

1. Introduction

Ketorolac (KR) is a potent non-steroidal anti-inflammatory and analgesic drug. Although HPLC/UV methods have previously been reported for KR [1,2], we required a more sensitive method for our studies and therefore evaluated GC/MS for the analysis of KR in plasma and other biological matrices. Gas chromatographic techniques coupled with selected-ion-monitoring (SIM) are ideally suited for the ultra trace analysis of KR, because they combine excellent sensitivity with a high degree of selectivity. However, in order to achieve accurate and precise quantitative measurements, they must be utilized with an internal standard (IS). The IS should be chosen to possess similar physicochemical properties to that of the analyte, especially comparable chromatographic retention behavior.

p-Fluoroketorolac (*P*-FKR) is similar in structure to ketorolac, differing only in the presence of

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a fluorine atom attached at the *para* position of the terminal benzene ring (Fig. 1). It has been evaluated in our laboratory as an IS for the determination of ketorolac in human plasma. In attempting to quantitate over a calibration range of 1–500 ng, we experienced difficulty in obtaining accurate results, particularly at the low end of the calibration range. We were able neither to correct nor improve the problem through extensive chromatographic and sample preparation modifications. As a potential solution, a stable isotope labeled [$^{18}O_3$]ketorolac ([$^{18}O_3$]KR, Fig. 1) derivative was synthesized and tested as a replacement for *P*-FKR. An apparent improvement in linearity of response resulted. To demonstrate unequivocally that the improvement achieved was indeed due to the nature of the IS, as opposed to some unrelated variable, an experiment was performed in which both IS candidates were added to identical ketorolac calibration standards and analyzed. Similarly, spiked plasma samples were prepared containing both ISs. These samples were used to measure method accuracy. Molecular ions were simultaneously monitored for the methyl ester of each of the three analytes. Two identical, yet separate sets of standards were prepared and analyzed, together with a set of plasma samples spiked with known amounts of KR. The resulting data facilitated a direct comparison of the two components for their suitability as ISs for the quantitative assay of KR in plasma.

2. Experimental

2.1. Reagents and materials

KR–tromethamine salt and *P*-FKR were obtained from Syntex Laboratories (Palo Alto, CA). [^{18}O]water (>98% atom pure) was purchased from Sigma/Aldrich (St. Louis, MO). Disposable columns (3 ml), coated with octadecylsilane and all solvents (HPLC grade) were obtained from J.T. Baker (Phillipsburg, NJ). Water used was distilled and further purified using a Sybron/Barnstead nanopure II system (Dubuque, IA). All other chemicals were reagent grade. Heparinized plasma for use in spiking experiments was obtained at our facility from volunteers. Stable isotope labeled [$^{18}O_3$]KR was synthesized in our laboratory by incorporating ^{18}O atoms into the KR molecule using an acid-catalyzed exchange procedure similar to that initially described by Murphy and Clay [3]. Briefly, 100 μg of KR was dissolved in a solution of acetonitrile:[^{18}O]water saturated with HCl gas (50:50; v/v) and reacted for 18 h at 50°C. The product was extracted with ethyl acetate and dried over anhydrous sodium sulfate. [$^{18}O_3$]KR was determined by chromatographic/SIM analysis to be >98% atom pure.

2.2. Preparation of calibration standards

Eighteen calibration standards, nine per set, in duplicate, were prepared by adding 1, 2, 5, 10, 20,

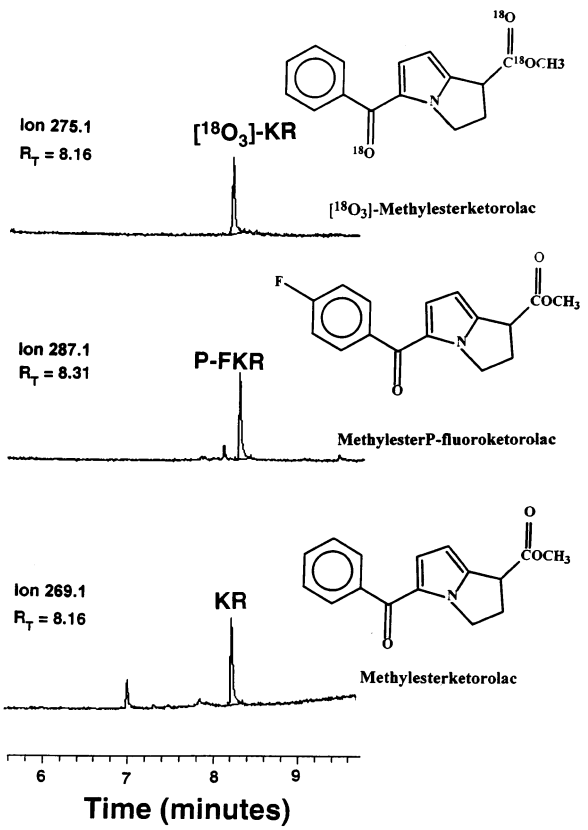

Fig. 1. Typical SIM chromatograms obtained for a plasma standard containing 10 ng/ml ketorolac, 10 ng/ml *p*-fluoroketorolac and 10 ng/ml [$^{18}O_3$]ketorolac, together with their corresponding chemical structures.

50, 100, 200 and 500 ng of KR dissolved in methanol, to individual autosampler vials. To each vial containing KR, an equal mass of *P*-FKR (10 ng) and [$^{18}O_3$]KR (~ 10 ng) was added. The solvent was removed in a nitrogen stream and the methyl esters were produced by reacting with ethereal diazomethane. Following the removal of ethereal diazomethane, each standard was reconstituted in 0.05 ml of decane and analyzed.

2.3. Preparation of spiked samples

Normal human plasma was filtered and 1.0 ml transferred to each of nine 16 × 100 mm disposable culture tubes. KR was added to each at one of the following levels: 2.0, 10.0 and 100.0 ng/ml ($n = 3$ at each level). Each sample was mixed and 10 ng of *P*-FKR and [$^{18}O_3$]KR was added to each sample in 10 μl of methanol. The samples were extracted using solid phase extraction (SPE) with octadecylsilane cartridges. Briefly, the entire sample was loaded onto an SPE cartridge that had previously been conditioned with 5 ml methanol followed by 5 ml of methanol/water (3/97, v/v). The cartridge was then washed sequentially with 5 ml each of methanol/water (3/97, v/v) and methanol/water (25/75, v/v). The cartridge was then eluted with 5 ml of methanol/water (75/25, v/v) and the eluant was taken to dryness under nitrogen at 30°C. The residue was reacted with ethereal diazomethane for several min. The solvent was again removed and the residue reconstituted with 50 μl of decane just prior to chromatographic analysis.

2.4. Apparatus and chromatographic conditions

The chromatographic system comprised a Model 5890A gas chromatograph, equipped with a model 7673A autosampler with tray (Hewlett-Packard, Palo Alto, CA). The GC was coupled with a model 5989A mass spectrometer (Hewlett-Packard). The methyl esters of KR, [$^{18}O_3$]KR and *P*-FKR were separated on a 30 m × 0.25 mm I.D. Rtx-5 (0.1 μm film thickness) fused silica capillary column (Restek, Bellefonte, PA). Helium was employed as the carrier gas at a column head pressure of 10 psi. The column temperature was ramped from 125°C to 300°C, at a rate of 20°C/min, following a 1 min hold at initial temperature. The split/splitless injector was operated in the splitless mode with the purge valve opened at 1.0 min after injection. A 4 mm deactivated straight cylindrical liner (Restek) packed with silanized glass wool was used in the injection port. The injection port was maintained at 250°C, while the transfer line temperature was 300°C. Molecular ions were simultaneously monitored at m/z 269, 275 and 287 for KR, [$^{18}O_3$]KR and *P*-FKR, respectively. Electronically integrated peak heights were obtained.

2.5. Data reduction

Separate peak height ratios (PHR, peak height KR/peak height IS) were calculated using each IS for every KR calibration standard. Weighted linear regression curves were constructed by regressing for each standard the PHR versus the mass, using weights equal to $(1/\text{mass})^2$. The concentration of KR in the spiked plasma samples was determined by interpolation from the linear regression curve.

3. Results

A typical chromatogram obtained for a KR calibration standard is shown in Fig. 1. KR is resolved from *P*-FKR and coelutes with [$^{18}O_3$]KR. Good peak shape was obtained, with only a slight degree of tailing. Typical PHRs obtained for injections of the KR calibration standards, using both *P*-FKR and [$^{18}O_3$]KR as the ISs, are shown in Table 1.

Typical weighted regression lines obtained using *P*-FKR and [$^{18}O_3$]KR as the ISs are depicted in Fig. 2. It can be seen that the points for the *P*-FKR based standard curve are further from the line than the points obtained for the [$^{18}O_3$]KR based curve. A log-log scale is depicted so as to more evenly distribute the points. Linear regression lines, when plotted on log-log scales, will appear linear over most, if not all, of the x-axis range. Such lines may deviate from linearity for

Table 1
Regression statistics weighted as $(1/\text{mass})^2$

	Obtained using *p*-fluoroketorolac		Obtained using [$^{18}O_3$]ketorolac	
	Coefficients	Standard error	Coefficients	Standard error
Intercept	−0.0152	0.049	0.0496	0.008
Slope	0.1719	0.018	0.0970	0.003
Coefficient of determination	—	0.926	—	0.993

Residuals Mass (ng)	Observed PHR	Predicted PHR	|% Residual|	Observed PHR	Predicted PHR	|% Residual|
1.02	0.190	0.160	15.7	0.157	0.149	5.3
2.03	0.268	0.334	24.6	0.214	0.247	15.2
5.08	0.658	0.858	30.4	0.537	0.543	1.0
10.2	1.40	1.74	24.2	1.05	1.039	1.0
20.3	2.77	3.47	25.4	1.98	2.02	2.0
50.8	7.52	8.72	15.9	5.08	4.98	2.0
101.6	18.8	17.5	7.2	9.97	9.91	0.6
203.2	42.8	34.9	18.4	19.7	19.8	0.3
508.4	129	87.3	32.4	53.2	49.3	7.2

small values of x, with the size of the deviation depending on the magnitude of the regression intercept term. Selected regression statistics corresponding to each regression plot are located in Table 1 and provide a quantitative comparison of the quality of the standard curves. The absolute value of the percent residuals, calculated using Eq. (1) below, clearly demonstrate the difference between the observed and the predicted PHR values, which are much larger for the *P*-FKR vs the [$^{18}O_3$]KR based standard curves. When these 'percent residuals' are plotted together (Fig. 3), they clearly demonstrate the fact that every point on the [$^{18}O_3$]KR plot is much closer to the regression line than its *P*-FKR counterpart. The calibration plots together with their corresponding regression statistics and residual analyses, dramatize the improvement in linearity that results when the [$^{18}O_3$]KR data is utilized.

$$\left|(\text{Predicted PHR} - \text{Observed PHR})/\text{Observed PHR} * 100\right|. \quad (1)$$

The improved calibration data is subsequently reflected in method accuracy as defined by the

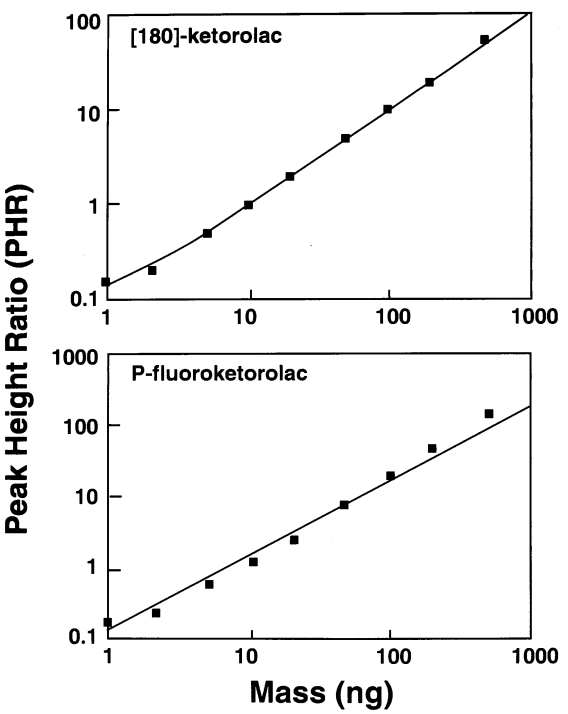

Fig. 2. Calibration curves for ketorolac, using [$^{18}O_3$]ketorolac (top) and *p*-fluoroketorolac (bottom) as internal standards.

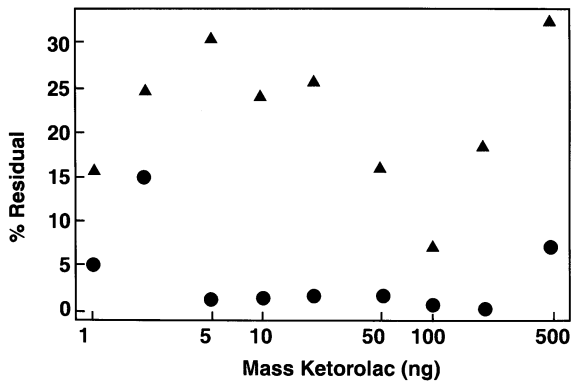

Fig. 3. A plot of the percent residuals, using [$^{18}O_3$]ketorolac (circle), and *p*-fluoroketorolac (triangle) as internal standards.

computed recovery of KR from spiked plasma samples (Table 2). The recovery data for KR, at all spiked plasma levels, were within 10% of the target value using [$^{18}O_3$]KR as the IS. Using *P*-FKR as the IS the KR recovery data were, in general, 19–30% lower than the target value. Interestingly, the precision obtained for replicate samples was similar, regardless of the IS used for the analysis.

4. Discussion

The divergent results obtained with the two *P*-FKR and [$^{18}O_3$]KR highlights the need to carefully select an IS for quantitative analysis when using gas chromatography. Although the structure of *P*-FKR is nearly identical to KR (see Fig. 1) it was not satisfactory as an IS for the quantitative analysis of KR. Differential recovery of KR and *P*-FKR during sample preparation could potentially account for these results. However, similar results were obtained for the injection of organic standards (data not shown). A potential explanation for the observed results is that KR undergoes a quasi-irreversible adsorption on active sites inside the capillary column. It is likely that the *P*-FKR also undergoes similar adsorption on the column, but since it is separated in time from KR, it does not correct for the adsorptive loss of KR. As a result, the peak height for KR increases disproportionately as the mass of injected KR increases due to saturation of the column active sites. That is, a larger signal is obtained as the mass of KR injected increases due to the fact that more of the KR is able to traverse the column to the detector. The [$^{18}O_3$]KR coelutes with KR, thereby correcting for the adsorptive loss of KR on the column by itself undergoing an identical adsorptive loss.

Table 2
Accuracy, as determined by recovery of spiked samples

Mass kerorolac spiked (ng)	Percent recovery, *p*-fluoroketorolac	Percent recovery, [$^{18}O_3$]ketorolac
2.0	78.6 ± 4.4	96.8 ± 2.7
10.0	67.2 ± 1.3	104.3 ± 1.2
100.0	81.3 ± 2.1	108.4 ± 2.0

5. Conclusions

In the case of the determination for ketorolac, a stable-isotope-labeled IS is necessary to accurately quantitate over the linear range examined. The differences observed for each IS as they relate to linearity of response as well as their effect on method accuracy has been documented. The observations reported conclusively indicate that the slight structural difference between KR and its close chemical relative, *P*-FKR, is significant enough to preclude its use as an internal standard for GC/MS analysis.

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