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Tissue extraction and high-performance liquid chromatographic determination of ketoprofen enantiomers

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Abstract

Local transcutaneous delivery of non-steroidal anti-inflammatory drugs avoids gastrointestinal side effects and concentrates drugs in the intended tissues. An extraction and HPLC method was developed for ketoprofen in skin, fascia and muscle. Tissue samples were homogenized in $NaHCO_3$. After methylene chloride removal of lipids, the aqueous layer was acidified with HCl and back extracted into isooctane/isopropanol. Ketoprofen was derivatized with ethylchloroformate/S-(−)-α-phenylethylamine in triethylamine, then detected by HPLC. Ketoprofen recovery was linear (1–33 μg/g) and was detected in these tissues following in vivo cathodic iontophoresis (160 mA∗min). This represents the first non-radioactive method for determination of ketoprofen in tissues following transcutaneous iontophoresis. © 1998 Elsevier Science B.V.

Keywords: Enantiomer separation; Ketoprofen

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are utilized for their anti-inflammatory effect in musculoskeletal dysfunction, and share a common propensity, induction of gastric or intestinal ulceration and possible bleeding [1]. These adverse gastrointestinal side effects have resulted in an increased focus on parenteral routes of administration. Transcutaneous NSAID permeation represents one parenteral route when localized musculoskeletal pain and inflammation require pharmacologic treatment.

Direct tissue permeation of NSAIDs have been investigated following in vivo passive and forced transcutaneous drug administration. Measurable concentrations of NSAIDs in serum and/or urine have been demonstrated following passive transcutaneous administration in both animals and humans [2–7]. Forced transcutaneous administration of NSAIDs has been governed by two different physical principles. The first principle uses mechanical ultrasound energy, phonophoresis/sonophoresis [8], and the second, electrochemical forces, iontophoresis/ionization/ionisation [4,9–11]. Following phonophoresis

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or iontophoresis concentrations of NSAIDs have been detected in the serum and/or urine of both animals and human subjects [4,8–10]. These results demonstrate systemic distribution following passive and forced transcutaneous NSAID administration.

The reported depth of direct transcutaneous tissue permeation following passive administration of NSAIDs varies. However, permeation down to or through the fascia has been documented for several other NSAIDs [6,12]. Passive administration of NSAIDs also reduces experimentally induced peripheral edema in animal models [13,14]. The maximal inhibition of edema was equivalent to oral delivery, and an ED_{50} 36% less than the oral dose on a per weight basis was required [14]. Thus, passive administration and transcutaneous permeation of NSAIDs results in measurable and anti-inflammatory concentrations of these drugs delivered to subcutaneous sites. However, determination of the pattern of transcutaneous tissue permeation and quantitation of the drug delivered have been limited to detection of radiolabeled NSAIDs within tissues [6,12], or detection of nonlabeled NSAIDs which reach systemic biologic fluids [2–5,7,9,10].

Ketoprofen is a NSAID with well documented chemical structure, pharmacokinetics and biochemical mechanism of action. Ketoprofen is racemic arylproprionic acid derivative [1,15], with approximately 120 times the anti-inflammatory potency of aspirin [16]. Pharmacologic activity resides almost entirely with the *S*-enantiomer, with only 10% of the *R*-enantiomer inverted to the antipode in humans. The average reported half-life for ketoprofen is 2.1 h. The therapeutic range of the anti-inflammatory effect for the *S*-enantiomer in the serum, and theoretically in the tissue, is 0.2–3 mg/l.

The purpose of the present investigation was to develop an analytical methodology to quantitate enantiomers of ketoprofen in skin, fascia and muscle, and determine if ketoprofen enantiomers are quantifiable following in vivo iontophoresis in these tissues. These tissues were chosen due to the previous documentation of transcutaneous permeation of other radiolabeled NSAIDs into these tissues following passive administration [6,12]. Ketoprofen was selected due to: (1) the documented anti-inflammatory effect in both an experimental animal model [14], and in humans [17] following passive transcutaneous administration; and (2) demonstrable levels of ketoprofen in venous circulation and urine following iontophoretic delivery in humans [9,10].

2. Experimental

2.1. Materials

Ketoprofen and fenoprofen, with a purity greater than 98% and a 50:50 '*R*' and '*S*' racemic mixtures, were obtained from Sigma (St. Louis, MO, USA). *S*-(−)-α-phenylethylamine and anhydrous isopropanol were also obtained from Sigma. Acetonitrile (Optima), isooctane, isopropanol, methylene chloride, methanol and glacial acetic acid were all HPLC grade. These HPLC reagents, $NaHCO_3$ and HCl were obtained from Fisher Scientific (Norcross, GA, USA). All borosilicate test tubes, Teflon sealed 50 and 25 ml tubes, 20 ml tubes and 30 ml polypropylene homogenization tubes were obtained from Fisher Scientific. Polypropylene tubes (250 μl) were from Cole Palmer Instrument (Niles, IL, USA).

2.2. Solution preparation

Standards of ketoprofen and fenoprofen (internal standard) were dissolved in methanol at 1 mg/ml and stored in Teflon sealed brown glass containers at 25°C. Ketoprofen utilized for in vivo iontophoresis was prepared at 300 mg/ml in phosphate buffered saline with 20% ethanol (v/v) as previously described [9,10]. The isooctane:isopropanol solution was prepared as a 95:5 (v/v) solution. The derivatization agents, 50 m*M* triethylamine and 6 m*M* ethyl chloroformate, were prepared in acetonitrile, and the derivatization agent *S*-(−)-α-phenylethylamine (0.5 *M*) was prepared in acetonitrile–triethylamine (8:2; v/v). All derivatization agents were stored in Teflon sealed brown glass containers at −20°C. The HPLC mobile phase was a mixture of acetonitrile–water–glacial-acetic acid–triethylamine (43:57:0.1:0.03, v/v). Water utilized for all solutions was 18 Ω eluent from a Barnstead Nanopure system (Dubuque, IA, USA).

2.3. Animal surgery and tissue sampling

All animal experimentation received prior approval from the institutional animal care and use commit-

tee. Surgical anesthesia in the pig was induced by atropine (0.05 mg/kg), ketamine (25 mg/kg), acepromazine (0.5 mg/kg) and sodium pentobarbital (15 mg/kg). Anesthesia was maintained by 0.5–1.0% halothane in oxygen. The animal was ventilated at 12–16 respirations per min and 300–400 ml per breath. The middle third of one medial thigh was chosen as the application site. The surface was prepared by removal of hair with clippers so as not to damage the stratum corneum, and precleaned with isopropyl alcohol swabs. Iontophoresis was conducted utilizing the medium EBIE applicator electrode and Dupel iontophoretic device from EMPI (Saint Paul, MN, USA). A total of 2.5 ml of the ketoprofen (750 mg) solution was applied to each applicator. The delivery applicator was placed on the skin. The applicator was attached to the cathodic electrode of the Dupel device, and the return anodic electrode placed on the abdomen approximately 25 cm from the delivery applicator. The iontophoretic dosage was 160 mA∗min (4 mA for 40 min). A 2.54 cm key hole drill bit, with the center mandril removed, was placed at the center of the iontophoretic application, and the circumference of the bit scribed with a scalpel. The skin underlying the electrode was then clipped from the underlying fascia and surrounding skin, and stored for analysis. The fascia was also clipped from the underlying muscle and stored separately for later analysis. Finally, a core sample of muscle was taken. The key hole drill was placed on the surface of the muscle, run in reverse to the lateral surface, and the muscle biopsy stored separately for later analysis. All samples were transported vertically on ice prior to storage (−80°C).

2.4. Sample preparation

Ketoprofen was extracted from skin, fascia and muscle. Spiked tissue was prepared by adding to each 30 ml round bottom polypropylene tube 0.5–10 μl of ketoprofen (1 mg/ml) and 40 μl fenoprofen (1 mg/ml). The standards were added followed by 0.5 g of skin, 0.5 g of fascia or 0.3 g muscle. Subsequently, 5 ml of 1 *M* $NaHCO_3$ was added and the tissue homogenized with a Omni International GLH tissue homogenizer (Gainesville, VA, USA) with 20× 195 mm stator generator and windows oversized to 1 mm. Tissue was homogenized until complete passage of the homogenate through a polypropylene pipet was obtained. Homogenization required 2–3 30 s repetitions with the homogenator on settings 2–4. The generator was washed between samples by a water, methanol–water (1:1, v/v), water sequence, and mechanically cleaned if required. Unless stated otherwise, samples and reagents were kept at 25°C during the subsequent processing. The homogenate was transferred to a 25 ml borosilicate round bottom tube. The 30 ml round bottom homogenization tube was rinsed twice with 5 ml methylene chloride and the rinses combined with the tissue homogenate. The borosilicate tube was Teflon capped, vortexed for 1 min, and centrifuged at 850 *g* for 40 min. The upper aqueous layer was pipetted into a 50 ml round bottom borosilicate tube, and solution acidified (pH=1.43±0.11) with 200 μl of 10 *M* HCl while vortexing. Next, 16 ml of isooctane:isopropanol was added, the sample vortexed 1 min, Teflon capped, and centrifuged (850 *g*, 20 min). The upper organic layer was transferred to a 20 ml borosilicate round bottom tube, and the organic solvent evaporated under a stream of N_2 at 70°C. Finally, the wall of the tube was rinsed with 0.5 ml of methanol, and evaporated to dryness again. Tissue samples were processed as above with the following differences: upon thawing, the skin and fascia samples were weighed and sufficient nonexposed tissue added to result in a final wet tissue weight of 0.5 g. Fenoprofen was added (40 μl, 1 mg/ml) and the samples subsequently processed as the standards. The muscle core was laid on a metal rule. Samples were obtained by slicing the core perpendicularly at 1 cm depths. Each 1 cm aliquot was weighed and sufficient 1 *M* $NaHCO_3$ added to make the final sample weight to volume ratio 0.1 g/ml. Each muscle sample was homogenized as above and 2.5 ml of the homogenate transferred to a 50 ml round bottom flask. An additional 2.5 ml of 1 *M* $NaHCO_3$ was added to make the final concentration 0.05 g/ml. Fenoprofen was added (40 μl, 1 mg/ml) to each muscle sample and processed as the standards.

2.5. Sample derivatization and high-performance liquid chromatographic (HPLC) detection

Sample derivatization and chromatographic separation and detection were conducted at 25°C. Concentrates were reconstituted in 100 μl of 50 m*M*

triethylamine, vortexed and 50 μl 6 m*M* ethylchloroformate added. The sample was incubated for 1 min, then 25 μl of 500 m*M* *S*-(−)-α-phenylethylamine was added, vortexed again and incubated for 4 min. The reaction was terminated by adding 50 μl water, vortexing and centrifugation (850 *g*, 25°C, 1 min). Samples were transferred to 250 μl polypropylene vials, injected via a 717 Autosampler, and analyzed on a HPLC which consisted of a M45 solution delivery system, Lambda Max Model 480 variable wavelength UV monitor, 746 data module, and Z-module, all from Waters (Milford, MA, USA). The 717 autosampler injected a 50 μl sample for analysis, and the injector rinsed with methanol–water (1:5, v/v) between samples. Analytes were eluted from a Waters Nova-Pak C_{18} radial compression column (8 mm×100 mm×4 μm) with 10 μm Bondapak C_{18} Guard-Paks (Waters), and a mobile phase flow-rate of 2.5 ml/min, and detected at 255 nm.

2.6. Calculations and statistical analysis

The peak areas under the curve for '*R*' and '*S*' diastereomers of ketoprofen or fenoprofen, internal standard, were added together for calculation of total ketoprofen or fenoprofen concentrations. Sample-to-sample variation was normalized by dividing the total ketoprofen peak area by the total fenoprofen peak area. Unless stated otherwise all data are reported as mean±standard deviation (S.D.). Regression analyses were calculated by plotting ketoprofen concentration on the abscissa and ketoprofen to fenoprofen peak area ratios on the ordinate. Significance of the regression function was determined from the coefficient of determination for linear regression (r^2) with $\alpha<0.05$. Calculations of unknown ketoprofen concentrations were determined by utilizing a linear regression function for each tissue type. All statistical analyses were conducted as previously described [10] utilizing SAS System for the PC (SAS Institute, Cary, NC, USA) on a 486/DX2 Gateway 2000 Nomad Notebook computer (North Sioux City, SD, USA).

3. Results

Ketoprofen and fenoprofen, internal standard, were extracted from all three tissues. Representative chromatograms of the diastereomers for ketoprofen and fenoprofen are illustrated following derivatization of standards without extraction, and following extraction from the three different tissues (Fig. 1). Retention times for the '*S*' and '*R*' isomers of

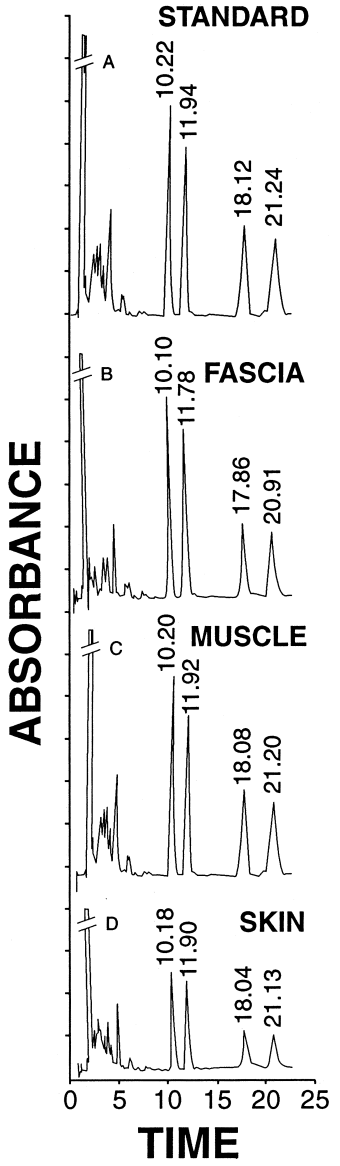

Fig. 1. Representative reversed-phase HPLC chromatograms of diastereomers of 5 μg ketoprofen and 40 μg fenoprofen, internal standard. Panels represent chromatograms of standards following no tissue extraction (A), or extraction from fascia (B), muscle (C), or skin (D).

Table 1
Regression parameters for ketoprofen tissue extraction

	Concentration Range[a]	Regression Function[b]	r^{2} [c]	N
Skin	0–20	$0.135x+0.046$	0.711*	42
Fascia	0–20	$0.258x+0.006$	0.888*	35
Muscle	0–33	$0.072x+0.032$	0.931*	30

Regression functions represent seven point standard curves conducted in six replications for skin and five replications for fascia, and six point standard curve conducted in five replications for muscle. All three coefficients of determination were significant (*).

[a] Total ($R+S$) ketoprofen concentrations were μg/gm wet weight tissue.

[b] Regression function is $y=m(x)+b$.

[c] Coefficient of determination for linear regression (r^2).

ketoprofen were 10.2 and 12.0 min, and 18.0 and 21.1 min for the '*R*' and '*S*' isomers of fenoprofen. Extraction of ketoprofen or the internal standard from the three different tissues did not appear to have any significant effect on the elution times (Fig. 1). The extraction, derivatization, and HPLC analysis of ketoprofen and fenoprofen as determined by peak area ratios were linear over the ketoprofen concentration ranges analyzed for each tissue type (Table 1). Regression coefficients for the three different tissues were significant at $\alpha<0.05$. The limit of detection for total ketoprofen was 1 μg/g in the skin, fascia and muscle.

Replicate analyses were performed for both between-day and within-day precision of ketoprofen extraction, derivatization and HPLC analyses from the three tissues (Table 2). For the various tissues, the coefficients of variation (C.V.) on the between-day replicates were greater for the ketoprofen concentrations at the lower range of each curve (9–10%), as compared to ketoprofen concentrations at the higher range of each curve (2–5%). Additionally, the between-day replicate C.V. for ketoprofen was comparable to the within-day replicate C.V. (1–4%). The extraction protocol was not stereoselective with respect to the '*R*' and '*S*' isomers of ketoprofen. The percentage of '*S*' ketoprofen isomer for 4 and 12 μg/g tissue, when extracted from skin, were 52±2% and 51±1% ($N=5$), and when extracted from fascia were 50±0.4% and 50±0.5% ($N=5$). The percentage of '*S*' ketoprofen isomer for 6.7 and 20 μg/g tissue when extracted from muscle were 51±1% and 51±2% ($N=5$).

Table 3
Ketoprofen tissue concentrations

Ketoprofen Isomer (μg/gm tissue)	'*S*'	'*R*'	Total
Skin	546	492	1038
Fascia	204	204	408
Muscle (cm)			
1	15.1	15.5	30.6
2	4.2	3.8	8.0
3	2.2	2.1	4.3
4	1.3	1.4	2.7
5	1.3	1.4	2.7

Depth of ketoprofen transcutaneous permeation following 160 mA∗min of cathodic iontophoresis to the medial surface of the pig thigh.

Total ketoprofen concentrations and percentage of '*S*' isomer were also determined in pig tissue following 160 mA∗min cathodic iontophoresis (40 mA, 40 min) to the medial surface of the thigh (Table 3). The '*R*' and '*S*' enantiomers of ketoprofen

Table 2
Total ketoprofen between-day and within-day statistics

	Ketoprofen concentration[a]	Between-day		Within-day	
		Mean±S.D.	C.V.[b]	Mean±S.D.	C.V.[b]
Skin	4	4.1±0.41	10	–	–
	12	12.4±0.26	2	12.1±0.26	2
Fascia	4	4.2±0.43	10	–	–
	12	12.1±0.52	4	12.3±0.16	1
Muscle	6.7	6.6±0.62	9	–	–
	20	20.9±1.1	5	19.3±0.80	4

Replications for between-day and within-day determinations were $N=5$ and $N=6$.

[a] Total ($R+S$) ketoprofen concentrations were in μg/gm wet weight tissue.

[b] Coefficient of variation (%).

were equally permeable in the three tissues examined. Ketoprofen concentrations decreased proportionately as tissue depth increased.

4. Discussion

Current research efforts are focused on the transcutaneous permeation of NSAIDs for treatment of localized musculoskeletal pain and inflammation, to avoid the adverse gastrointestinal effects of oral delivery. Previous research has demonstrated passive transcutaneous permeation of NSAIDs down to or through the fascia [6,12]. Transcutaneous permeation of NSAIDs has resulted in beneficial outcomes in both animal models of inflammation [13,14] and under clinical conditions [17]. However, previous methodologies have limited research efforts utilizing either radiolabeled NSAIDs in tissues [6,12] or detecting unadulterated drugs in serum or urine [2–5,7,9,10]. Thus, the present investigation examined the potential for determining whether unlabeled NSAIDs could be detected at therapeutic concentrations in tissues where these agents may permeate following transcutaneous application. Ketoprofen was selected as a representative NSAID due to the demonstrated potential of ketoprofen for transcutaneous permeation [2,5,9,10] and the experimental [14] and clinical evidence [17] of the effectiveness of the drug following transcutaneous permeation.

The current protocol for the extraction, derivatization and HPLC determination of ketoprofen enantiomers from skin, fascia and muscle was the culmination of several procedures with the following specifically noted limitations and caveats. The weight of tissue utilized for the current methodology was 0.5 g for skin and fascia and 0.3 g for muscle. When all reagents were held constant, the use of 1 g or heavier starting tissue weights resulted in lower yield of ketoprofen and fenoprofen. For muscle the starting weight was further decreased from 0.5 g to 0.3 g to increase yield. Whether ketoprofen yield would have increased with lower starting tissue weights was not examined. However, lowering starting tissue weight results in an inverse relationship to the minimal detectable ketoprofen concentration.

The tissue homogenization with $NaHCO_3$ and lipid extraction with methylene chloride were also examined with several different reagents. Both NaOH and NH_4OH were examined as initial homogenization buffers. Homogenization of tissue samples in the latter bases resulted in lower HPLC detection of the enantiomers for both ketoprofen and fenoprofen. The mechanism for the lower yields is unknown. However, during the acidification step addition of HCl resulted in notable precipitate with bases other than $NaHCO_3$. Whether the precipitation resulted in trapping of ketoprofen and fenoprofen has not been determined. During acidification with HCl, the precipitate problem is avoided with $NaHCO_3$ as gaseous CO_2 is evolved.

Methylene chloride was chosen to remove lipids due to its specific gravity greater than 1 g/ml and the insolubility of the organic in $NaHCO_3$. The heavy specific gravity of the organic provided both good separation from the aqueous phase following centrifugation, access to the upper aqueous phase post centrifugation, and excellent lipid extraction. Following addition of methylene chloride all samples were maintained at room temperature. If samples were cooled following addition of methylene chloride, the recovery of both ketoprofen and fenoprofen was lower. The mechanism for the lower yield was not investigated, but may have resulted from the partitioning of the ketoprofen and fenoprofen into the organic phase at the cooler temperatures.

A limited number of samples could be processed at any one time. Due to the homogenization and multiple isolation steps, the maximum successful number of samples that could be processed by one investigator was 46. During the derivatization phase of the protocol, a 1 min incubation step exists between addition of ethylchloroformate and *S*-(−)-α-phenylethylamine. The 1 min incubation limited the number of samples processed at this point to 24. The authors also observed that the tissue extraction, derivatization and HPLC detection was a two-day process, with tissue extraction conducted the first day and derivatization and HPLC detection the second day.

Stereospecific detection of *R* and *S* enantiomers of ketoprofen and fenoprofen, internal standard, resulted in equivalent detection of the enantiomers for both compounds. Previous investigations utilizing the current stereospecific methodology for detection

of enantiomers of ketoprofen and fenoprofen in serum and urine also demonstrated equivalent detection of *R* and *S* enantiomers, and with similar elution profiles [9,10,18]. Jamali and Brocks [15] in an extensive review of previous investigations determining stereospecific ketoprofen concentrations in various body fluids, but not tissue, also demonstrated equivalent detection of *R* and *S* enantiomers. Thus, the extraction and derivatization methodology utilized in the present investigation was not stereoselective to one ketoprofen enantiomer, and these results confirm previous investigations utilizing the present derivatization protocol and other investigations utilizing various stereospecific methodologies for the detection of ketoprofen enantiomers.

The precision, as measured by coefficient of variation (C.V.), for the current extraction and detection of ketoprofen from various tissues was below 10% for between-day precision and 4% for within-day precision in all three tissues analyzed. Utilizing the current derivatization and detection methodology, the C.V. for the extraction and detection of ketoprofen enantiomers from serum and urine was below 10% in previous investigations [10,18]. Thus, the precision in the present investigation agrees with previous investigations utilizing these methods.

Various published HPLC stereo-specific methods for ketoprofen determination have been reviewed and report an average of 0.1 μg/ml in body fluids [15]. The limit of detection for total ketoprofen in the present investigation was 1 μg/g tissue in skin, fascia and muscle. Investigations utilizing the present derivatization methodology have reported a detection limit for total ketoprofen as 0.05 μg/ml in plasma [18] and 0.25 μg/ml in urine [10]. The current authors have utilized the present derivatization methodology following extraction of ketoprofen from serum and urine and obtained a detectable limit of 0.3125 μg/ml (data not shown). The higher limit of detection for total ketoprofen in the present investigation was due to tissue matrix effects during the extraction of ketoprofen, not the efficiency of the derivatization of the enantiomers or their UV detection.

No endogenous compounds in the tissues were observed to co-eluate with the *R* and *S* enantiomers of ketoprofen or fenoprofen. Other investigations [9,10,18] and unpublished work by the current authors obtained similar results for the *R* and *S* enantiomers of ketoprofen and fenoprofen when determined in serum and urine. Thus, the current stereospecific determination of ketoprofen concentrations in tissue results in similar precision and selectivity, but a slightly greater limit of detection.

The value of the current methodology for stereospecific extraction and determination of ketoprofen during in vivo transcutaneous NSAID iontophoresis was demonstrated. Human systemic serum concentrations following repetitive applications resulted in passive ketoprofen permeation from different regions of the body that ranged 0.09–0.15 μg/ml [5]. Repetitive application of ketoprofen gel to the human knee resulted in passive transcutaneous permeation of ketoprofen down to the synovium with synovial fluid concentrations attaining 1.3 μg/ml [2]. Additionally, iontophoretic transcutaneous ketoprofen permeation resulted in 0.88 μg/ml in the local venous washout from the tissue. These results reviewed in aggregate support the value of attempting to quantitate NSAIDS in tissue following transcutaneous permeation. Finally, the derivatization method utilized in the present investigation has been demonstrated to successfully dimerize the enantiomers of the following NSAIDs: ibuprofen, naproxen, etodolac and tiaprofenic acid [18]. The present tissue extraction methodology should also be successful with these same NSAIDs. In conclusion, due to the similarities in derivatization and HPLC detection for the above anionic NSAIDs, the present results demonstrate a non-radioactive extraction and derivatization methodology for stereospecific determination of anionic NSAIDs from significant tissues related to transcutaneous delivery of these pharmacologic agents.

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