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Rapid online-SPE-MS/MS method for ketoprofen determination in dermal interstitial fluid samples from rats obtained by microdialysis or open-flow microperfusion

Karin E. Pickl [a], Christoph Magnes [a], Manfred Bodenlenz [a], Thomas R. Pieber [a,b], Frank M. Sinner [a,*]

[a] *Institute of Medical Technologies and Health Management, Joanneum Research, Auenbruggerplatz 20/3, 8036 Graz, Austria*
[b] *Medical University Graz, Department of Internal Medicine, Division of Diabetes and Metabolism, Auenbruggerplatz 15, 8036 Graz, Austria*

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Abstract

Pharmacokinetic studies of topical ketoprofen formulations using continuous sampling techniques such as microdialysis (MD) or open-flow microperfusion (OFM) require sensitive assays due to small sample volumes. A simple and easy online-SPE-MS/MS method for ketoprofen analysis was developed for both MD and OFM samples obtained from rat dermal tissue. The quantification range is 25–5000 ng/ml with a limit of detection of 3 ng/ml using only 10 μl sample volume. The method is characterized by a simple setup using a short polymeric SPE column (OASIS HLB) for desalting with 1.5 min run times in combination with a sensitive MS detection in negative ESI MRM mode. An easy sample workup procedure was used which enables high throughput analysis of a large number of samples for pharmacokinetic studies. In addition, a commercial available (fenoprofen) as well as an isotopically labelled (deuterated ketoprofen) standard were investigated as potential internal standards. The method was validated according to FDA guidelines for bioanalytical validation in terms of accuracy, intra-batch and inter-batch precision, linearity, matrix effect, recovery and stability for both internal standards. Accuracies were 98–113% (fenoprofen) and 95–108% (deuterated ketoprofen), intra-batch precision was 2–3% R.S.D. (fenoprofen) and 2–6% R.S.D. (deuterated ketoprofen), and inter-batch precision was 2–6% R.S.D. (fenoprofen) and 3–6% R.S.D. (deuterated ketoprofen) over the entire quantification range. The presented method was applied to dermal interstitial fluid samples obtained in a topical administration study of ketoprofen in rats.
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1. Introduction

Topical application of certain drugs is a good alternative to oral administration for certain active substances, avoiding unwanted systemic side effects. Ketoprofen is a non-steroidal anti-inflammatory drug (NSAID) which is generally very effective for the relief of pain and inflammation. However, if orally administered, it can lead to several adverse effects that primarily involve the gastrointestinal tract and the kidneys [1]. For this reason, much effort is being put into the development of ketoprofen formulations for topical administration which efficiently enhance its transport through the skin into the body [2].

In order to investigate the efficiency of different topical formulations of ketoprofen in vivo, the pharmacokinetics of ketoprofen need to be quantified. Therefore, continuous sampling of dermal interstitial fluid from skin tissue over a longer period is necessary. Microdialysis (MD) and open-flow microperfusion (OFM) are continuous sampling techniques that enable in vivo investigations of events in interstitial fluid (ISF) of various tissues including dermal tissue [3–5].

Microdialysis is a well established technique for the assessment of dermal drug delivery but has certain drawbacks associated with the use of a semipermeable membrane such as low recoveries for large molecules and protein-bound drugs and difficulties with sampling of highly lipophilic drugs [3]. With open-flow microperfusion, analytes are sampled through macroscopic holes which enables direct access to interstitial fluid without a membrane. Sampling of large molecules such

* Corresponding author. Tel.: +43 316 876 2103; fax: +43 316 876 2104.
E-mail address: frank.sinner@joanneum.at (F.M. Sinner).

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as insulin [6] and albumin [7] is thus facilitated and there is a high potential for dermal sampling of lipophilic or highly protein-bound drugs like ketoprofen.

ISF samples put special demands on the analytical technique: MD and OFM use low flow rates (down to 0.3 μl/min), yielding small sample volumes ranging from 2 to 100 μl depending on the sampling time. In addition, if the ion reference technique [5] – a calibration technique in MD and OFM experiments – is applied, multiple analytes need to be quantified from the same ISF sample with different analysis methods (e.g. ketoprofen using LC/MS and sodium using F-AAS). Furthermore, ISF concentrations are generally lower compared to plasma concentrations [6,7] and in addition, the sampling techniques usually deliver diluted ISF which further reduces the analyte concentration in an ISF sample. As a consequence, highly sensitive assays for small sample volumes are needed for performing microdialysis and open-flow microperfusion studies.

There is a large number of analytical methods for ketoprofen determination in plasma using either HPLC-UV [8–10], GC–MS [11], SFC-MS [12] or HPLC-MS [13,14] which typically require sample volumes from 200 up to 1000 μl and are thus not suitable for the analysis of interstitial fluid samples from MD or OFM. No publications to date have focused on the development of an analysis method for ketoprofen in MD or OFM samples. However, two papers on microdialysis studies of ketoprofen do include limited information on the method of analysis used. He et al. [15] used an isocratic HPLC-UV method for ketoprofen analysis in blood microdialysis and plasma samples with run times of 8 min. Twenty microliters extract from 200 μl plasma is injected, however the authors did not state how much microdialysis sample is needed for direct injection into the HPLC-UV system. The second paper by Tegeder et al. [16] used an LC/MS method validated in plasma and ultrafiltrated plasma. Twenty microliters microdialysis samples were diluted with labelled ketoprofen (internal standard) to a volume of 70 μl and then 35 μl were injected for LC/MS analysis. However, even sample volumes of 20 μl can be difficult to obtain for analysis as mentioned above. Moreover, microdialysis provides relatively clean and almost protein-free samples which are commonly analyzed without further clean-up procedures [15,16]. In contrast, samples acquired by open-flow microperfusion are expected to contain a higher amount of proteins owing to stable sampling of large molecules such as albumin, as has been reported in the literature [7].

Thus, to further broaden the potential of the microdialysis and open-flow microperfusion technique for clinical studies on ketoprofen formulations in dermal tissue in terms of study design (e.g. lower flow rates, shorter sampling periods, catheter choice (MD or OFM), application of ion reference technique), a sensitive and reliable ketoprofen assay suitable for both MD and OFM samples is needed.

We report a simple online-SPE-MS/MS method for ketoprofen analysis from 10 μl MD and OFM samples obtained from rat dermal tissue. The method is further characterized by a simple setup using a short polymeric SPE column (OASIS HLB) for desalting with run times of only 1.5 min in combination with sensitive MS detection in negative ESI MRM mode. A simple sample workup procedure including protein precipitation was used which enables high-throughput analysis of a large number of samples from pharmacokinetic studies. To further broaden the applicability of this new method, we made use of a commercially available standard (fenoprofen) as an internal standard, which was evaluated against a non-commercially available isotopically labelled standard (deuterated ketoprofen). The presented method was validated according to FDA guidelines for bioanalytical validation [17] in terms of accuracy, intra-batch and inter-batch precision, linearity, matrix effect, recovery and stability for both investigated standards. Fenoprofen as well as deuterated ketoprofen were found to be suitable as internal standards with a calculated limit of detection of approximately 3 ng/ml and a quantification range from 25 to 5000 ng/ml.

The presented method was successfully applied to a topical administration study of ketoprofen in dermal tissue of rats.

2. Materials and methods

2.1. Chemicals and reagents

Ketoprofen (>98%), fenoprofen (>97%), water (for HPLC), acetonitrile (>99.9%, for HPLC), ammonium hydroxide (25% aqueous solution), methanol (>99.9%, for HPLC) and albumin from human serum (96–99%) were purchased from Sigma–Aldrich Handels GmbH (Vienna, Austria). 0.9% sodium chloride solution was obtained from Mayrhofer Pharmazeutika (Linz, Austria). Deuterated ketoprofen was synthesized according to Leis et al. [11].

2.2. Blank interstitial fluid

Blank interstitial fluid from rat skin was obtained by pooling samples obtained during a microdialysis and open-flow microperfusion study performed at JSW Research Forschungslabor, Graz.

2.3. Preparation of calibration standards and QC samples

Stock solutions of ketoprofen (KET) and fenoprofen (FEN) were prepared in methanol and diluted with water to their final concentrations (KET 0, 10, 39, 156, 625, 2500 and 10,000 ng/ml, FEN 400 ng/ml). Deuterated ketoprofen (dKET) was prepared in methanol and diluted with methanol to 400 ng/ml. Calibration of ketoprofen was performed by establishing a linear regression function after $1/X$ weighting of the analyte/I.S. peak area ratio versus analyte concentration relationship.

QC samples were prepared by successive dilution of a second stock solution of KET in a substitute matrix (1A) which was a 1% human serum albumin and 0.9% sodium chloride solution. The calibration standards, internal standards and QC samples were aliquoted and stored at −80 °C.

2.4. Sample processing

Samples were thawed and mixed shortly prior to sample processing. Ten microliters dKET (dissolved in 100% methanol, precooled to −80 °C), 10 μl FEN and 100 μl water were added to 10 μl samples in a PCR tube. The mixture was mixed and

incubated at 90 °C for 15 min. After incubation, the mixture was centrifuged at 13,000 × *g* for 5 min. One hundred microliters supernatant was transferred to a 96 well plate and further diluted with 100 μl water. Calibration standards were processed as described above but without incubation.

2.5. Online-SPE

All experiments were carried out on an Ultimate System (Dionex, LC Packings) including a FAMOS autosampler with cooled tray (4 °C) and a SWITCHOS loading pump coupled to a TSQ Quantum Ultra AM mass spectrometer (Thermo Finnigan). The system was controlled by Xcalibur Software 1.4. Fifty microliters of processed sample was loaded onto an OASIS HLB SPE column (2.1 mm × 20 mm, Waters) with water at a flow rate of 500 μl/min using the SWITCHOS loading pump (=loading mode) for desalting. After 0.5 min, the column was switched to the elution mode using the Ultimate pump with 80:20 acetonitrile:water, containing 0.025% ammonium hydroxide as mobile phase at a flow rate of 500 μl/min. At 1.4 min, the column was switched back to the loading mode. After 1.5 min, the system was ready for the next injection. Chromatography was performed at ambient temperature.

2.6. MS parameters

Detection was performed in negative ESI MRM mode using the following parameters: spray voltage 4.5 kV, sheath gas pressure 10 AU, auxiliary gas pressure 20 AU, capillary temperature 370 °C. The optimized collision energy for MRM was 10 eV with a collision gas pressure of 0.5 mTorr. The selected reactions for quantification were as follows: 253 → 209 *m/z* (KET), 241 → 197 *m/z* (FEN) and 260 → 216 *m/z* (dKET).

2.7. Recovery and matrix effect

Recovery (RE) and matrix effect (ME) experiments were performed in triplicate at two different KET concentrations (100 and 5000 ng/ml) for the following matrices: substitute matrix (=1A), dermal interstitial fluid from rats obtained with either microdialysis (=MD-ISF) and open-flow microperfusion (=OFM-ISF). For assessing RE and ME, three sets of samples were prepared according to Matuszewski et al. [18]. In Set A the matrix was spiked with KET prior to sample processing, in Set B blank matrix was processed without the addition of internal standards and spiked with KET, FEN and dKET after processing. Set C was analogous to Set B but used water as matrix. Recoveries (RE) for KET and the internal standards dKET and FEN were calculated by the peak area ratio of Set A to Set B multiplied by 100%. The matrix effect (ME) was obtained by the peak area ratio of Set B to set C multiplied by 100% (ion suppression if ME < 100%, ion enhancement if ME > 100%).

2.8. Accuracy and precision

QC samples with 25, 50, 100, 200, 1000, 5000 ng/ml KET were prepared in solution 1A, processed and analyzed five times in the same batch for intra-batch precision and once in five separate batches on different days for inter-batch precision. The accuracy was calculated for each concentration as the ratio of the measured concentration to the nominal concentration multiplied by 100%. The deviation of accuracy should be <15% and precision <15% [17].

2.9. Limit of quantification

The LoQ was calculated from the calibration curves (area ratio of zero standard multiplied by 5) to be approximately 25 ng/ml. During method validation, a QC sample with 25 ng/ml KET was processed and analyzed five times in the same batch and once in five separate batches at different days. Accuracy should range between 80 and 120% and precision should be <20% at LoQ [17].

2.10. Stability

The stability of KET was tested in triplicate for different storage temperatures (room temperature, +4 and −80 °C) for 1 week and for three freeze-thaw cycles at two different concentrations (200 and 5000 ng/ml). The results were compared to an aliquot of the same solution processed immediately after thawing. Stability was assured if the difference was below 10%.

2.11. Application of the online-SPE-MS/MS method

The presented online-SPE-MS/MS method was applied to analyze samples from an open-flow microperfusion study in dermal tissue of rats for the pharmacokinetic comparison of different topical ketoprofen formulations (data not shown). Briefly, four open-flow microperfusion sampling probes for dermal use (o.d. 0.4 mm) were inserted into the dermal layer of the skin of anesthetized Wistar rats and continuously perfused with ion-free isotonic solution (5% mannitol in aqueous solution) at a nominal flow rate of 1 μl/min. Ketoprofen (2.62% solution) was applied to defined areas above the inserted sampling probes (1 area above 2 sampling probes each, 2 areas with 16.5 mm i.d. (2.14 cm^2) per rat, 254 μl solution per area). Probe effluents were collected in 20 min fractions and were immediately frozen and stored at −80 °C until analysis.

3. Results and discussion

The extraction of dermal interstitial fluid via microdialysis and open-flow microperfusion is a complex and laborious procedure. Obtaining higher amounts of dermal interstitial fluid for method development and validation, particularly from rats, is difficult. For this reason, most of the method development and validation was performed with a substitute matrix that reflects the same characteristics as dermal interstitial fluid obtained with MD or OFM: high ionic strength and low amounts of protein. One percent of human serum albumin in 0.9% sodium chloride solution (1A) was therefore chosen as the substitute matrix. Only critical parameters such as selectivity, extraction recovery,

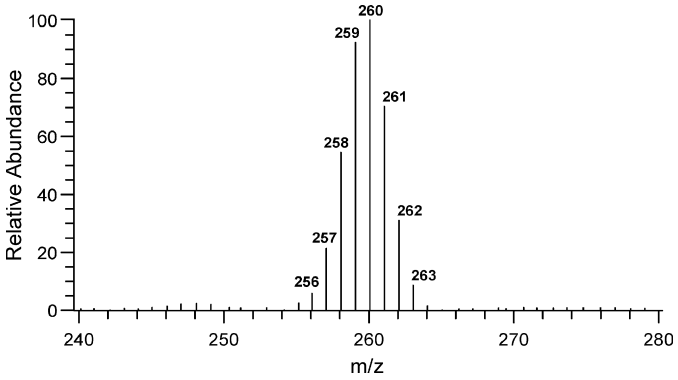

Fig. 1. Negative ESI spectrum of deuterated ketoprofen (d^2–d^9) obtained by direct loop injection in 80:20 acetonitrile:water, containing 0.025% ammonium hydroxide at 500 μl/min.

matrix effect and accuracy were additionally verified with small samples obtained from rats.

3.1. MS conditions

The mass spectrometer was operated in negative ESI mode. The ESI mass spectra of ketoprofen and fenoprofen showed one abundant peak corresponding to the deprotonated molecular ion $[M–H]^-$ at *m/z* 253 (KET) and *m/z* 241 (FEN). For the deuterated ketoprofen, several $[M–H]^-$ ions corresponding to the different labelling states of ketoprofen were observed ranging from *m/z* 255 (d^2-KET) up to *m/z* 262 (d^9-KET) with the most intensive ions being *m/z* 259 (d^6-KET) and *m/z* 260 (d^7-KET) (Fig. 1). Unlabelled ketoprofen was not observed in the dKET standard (Fig. 1). This corresponds well to data published on the preparation of the dKET standard [11].

The loss of carbon dioxide (*m/z* = 44) was chosen as a specific reaction for the MRM mode, both for ketoprofen and the internal standards used. Fig. 2 shows the corresponding product ion spectra. For deuterated ketoprofen, the MRM reaction 260 → 216 corresponding to the most abundant labelling state, d^7-KET, was monitored. The optimum conditions for the MRM mode with the mass spectrometer used were found to be 10 eV for collision energy and 0.5 mTorr for the collision gas pressure.

3.2. Online-SPE conditions

In order to develop a very fast method, we used an online-SPE step prior to MS detection. As the elution was performed at basic pH, a high pH-resistant polymer-based SPE column (OASIS HLB) was used. After 0.5 min of desalting with 100% water, KET and the internal standards FEN and dKET were immediately eluted at approximately 0.7 min using a mobile phase containing 80% acetonitrile. After only 1.5 min, the system was ready for the next injection which enabled complete analysis of one 96-well plate in duplicate injections in less than 5 h.

3.3. Selectivity

Online-SPE is basically a desalting step prior to MS detection. In order to ensure the absence of interference due to online-SPE, chromatographic profiles from injections of pure

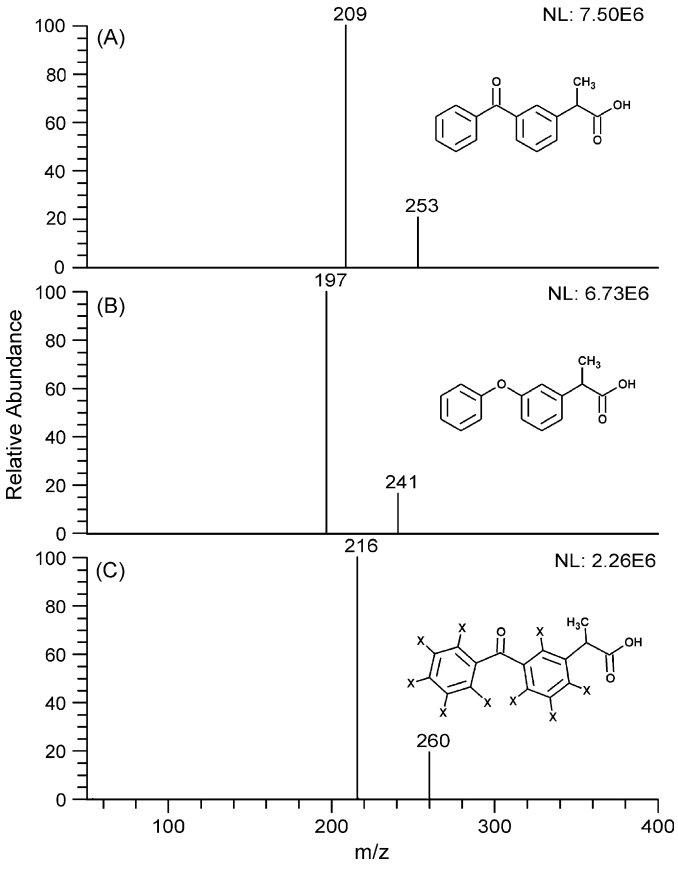

Fig. 2. Product ion spectra of (A) *m/z* 253, ketoprofen; (B) *m/z* 241, fenoprofen and (C) *m/z* 260, d^7-labelling state of deuterated ketoprofen (X marks all possible labelling positions, $X_9 = D_7H_2$) at CE 10 eV and 0.5 mTorr.

water, QC controls and processed blank matrices were compared. Small peaks monitored at the retention times of KET, FEN and dKET could be observed in all blank chromatographic profiles and even in pure water injections (Fig. 3A). This could be explained by the dramatic change of the mobile phase from 0 to 80% acetonitrile during switching from sample loading to sample elution leading to rapid elution of possible contaminants from the SPE column. However, no significant difference between water matrix and ISF matrices (Fig. 3) could be observed.

Blank interstitial fluid from rat skin tissue obtained with microdialysis (MD-ISF) and open-flow microperfusion (OFM-ISF) as well as the substitute matrix (1A) were each processed and analyzed three times with the presented online-SPE-MS/MS method and all measured ketoprofen concentrations were below calculated LoD (refer Fig. 3).

3.4. Sample processing

For analysis of a high number of samples for pharmacokinetic studies in a reasonable time frame, the sample processing should be as simple, fast and cost effective as possible. Analysis of MD samples is often performed without any sample processing [15,16,19] because MD samples generally have a very low protein content due to the passage of the sample through a

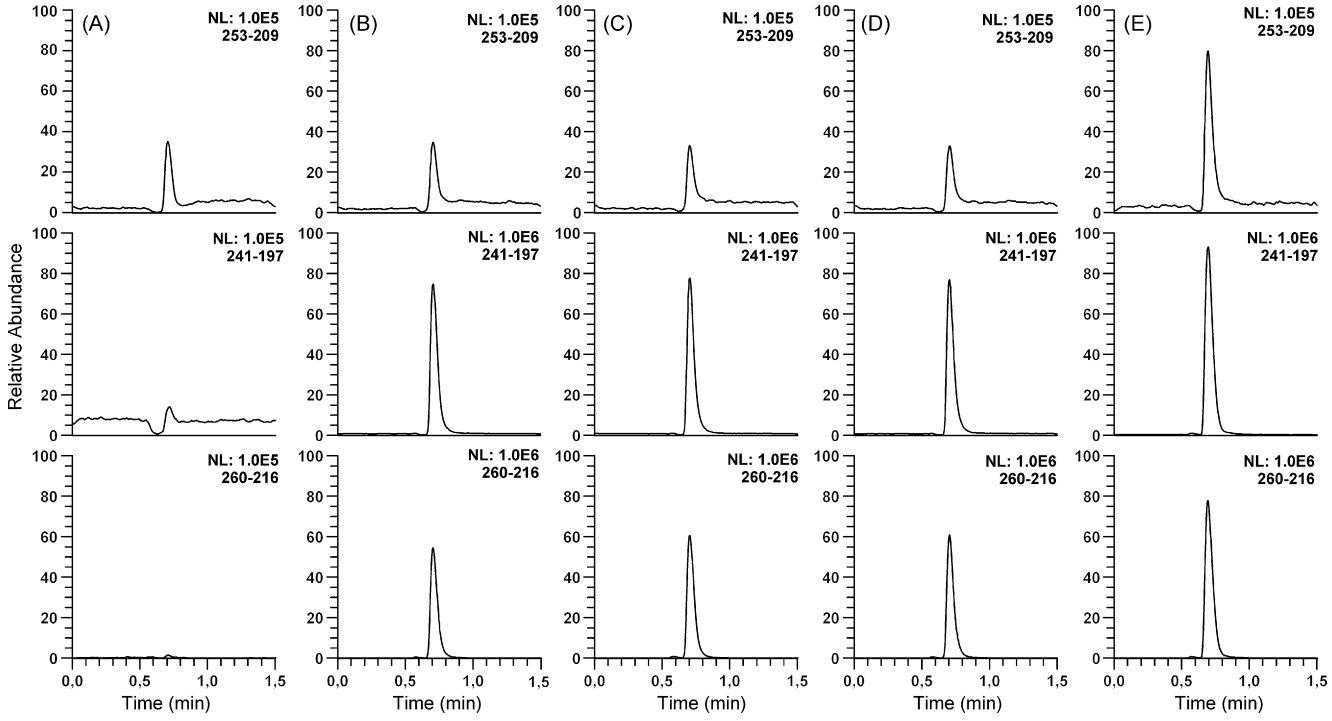

Fig. 3. Online-SPE-MS/MS chromatographic profiles of water (A), blank substitute matrix 1A (B), blank MD-ISF (C), blank OFM-ISF (D) and a 25 ng/ml QC control (E).

membrane. However, for open-flow microperfusion sampling is performed through macroscopic holes, resulting in the presence of considerable amounts of proteins. As the developed method should be suitable for both MD and OFM samples, we used protein precipitation for protein removal because it is a simple procedure even for small sample volumes. After protein precipitation, samples were centrifuged and the supernatant was further diluted, due to the high sensitivity of the MS detection used. Therefore, from 10 μl original sample up to four injections are possible. For comparison, the method by Tegeder et al. only allows up to two injections from 20 μl original sample. Sample processing recoveries were determined for rat dermal interstitial fluid from microdialysis (MD-ISF) and open-flow microperfusion (OFM-ISF) as well as for the ISF substitute matrix (1A) at low and high KET concentrations (100 and 5000 ng/ml).

The applied protein precipitation protocol resulted in recoveries of 93–108% for KET, FEN and dKET (Table 1). Recoveries were constant at low and high KET concentrations and relative standard deviations (%R.S.D.) were between 2 and 6% for all matrices investigated which indicates reproducible recovery during sample processing. No difference could be observed in recovery between the substitute matrix 1A and the original ISF matrices MD-ISF and OFM-ISF. This indicates that the chosen substitute matrix reflects the same characteristics as ISF.

Table 1
Average recovery, % (%R.S.D.) for different matrices and concentrations

Matrix	Conc KET (ng/ml)	Mean recovery (%)[a]		
		KET	FEN	dKET
1A	100	102 (4)	96 (4)	102 (5)
1A	5000	104 (2)	94 (1)	104 (1)
MD-ISF	100	100 (4)	93 (4)	98 (6)
MD-ISF	5000	105 (3)	100 (3)	103 (2)
OFM-ISF	100	108 (2)	98 (4)	107 (4)
OFM-ISF	5000	106 (4)	97 (3)	103 (4)

[a] $n = 3$ per matrix and concentration, calculation of recovery see Section 2.

3.5. Matrix effect

The matrix effect is a particular issue in LC–MS/MS analysis [18] which has gained attention in recent years [17]. Even though the matrix of interstitial fluid samples is considered to be less critical than other matrices such as plasma because of its low protein content, matrix effects are reported in the literature [19]. For this reason, we investigated a possible matrix effect with the presented online-SPE-MS/MS method in different matrices including MD-ISF, OFM-ISF and the substitute matrix 1A and whether this would influence the quality of the results.

The comparison of ketoprofen peak areas in standards and matrix revealed a certain ion suppression (matrix effect < 100%) for all matrices investigated. The average calculated matrix effect ($n = 6$) is comparable for all three matrices investigated with (81 ± 2)% for 1A, (85 ± 3)% for MD-ISF and (79 ± 1)% for OFM-ISF which further shows that solution 1A is a good choice for ISF substitute matrix for method validation. Interestingly, no difference in matrix effect could be detected between MD-ISF and OFM-ISF matrix even though ISF obtained from open-flow microperfusion generally has higher protein concentrations due to the method of sampling through macroscopic holes. Higher amounts of protein in OFM-ISF samples thus appeared to have been efficiently removed during sample processing.

Table 2
Average calibration data, correlation coefficients and calculated LoD obtained during method validation ($n = 9$ batches)

I.S.	$y = a$ (S.D.) + xb (S.D.)	Corr coeff = r^2 (S.D.)	LoD[a]
FEN	$y = 0.018$ (0.006) + $x0.0023$ (0.0001)	0.9994 (0.0008)[b]	2.4
dKET	$y = 0.024$ (0.009) + $x0.0031$ (0.0002)	0.9998 (0.0001)	2.6

[a] In ng/ml, calculated from the calibration curve, blank response + 3 × S.D. of blank response.
[b] All correlation coefficients >0.9995 except one with 0.9972.

Table 3
Accuracy, intra-batch and inter-batch precision for FEN and dKET as I.S. in matrix 1A ($n = 5$).

Nominal conc (ng/ml)	Accuracy within a batch, % (intra-batch precision, %)		Accuracy between batches, % (inter-batch precision, %)	
	FEN	dKET	FEN	dKET
25 (LoQ)	113 (3)	104 (2)	110 (6)	107 (6)
50	111 (3)	108 (4)	107 (3)	103 (3)
100	105 (2)	103 (3)	105 (4)	100 (5)
200	104 (3)	102 (2)	104 (4)	101 (6)
1000	103 (3)	104 (3)	104 (3)	100 (5)
5000	99 (2)	101 (6)	98 (2)	95 (3)

Although the presence of a matrix effect may be of some concern, of greater importance is the evaluation of whether the internal standard is affected in the same way as the target analyte [18]. As a consequence, the analyte/I.S. peak area ratio would remain unaffected. Fig. 4 summarizes the observed matrix effects for KET, FEN and dKET in all study matrices. Within a given matrix, KET and the two internal standards showed a matrix effect to the same extent. Furthermore, no significant differences between the tested matrices were observed. Small variations between the matrix effect of KET, FEN and dKET within a given matrix could be explained by the spiking procedure for the matrix effect test which involved a relatively high number of pipetting steps with small sample volumes (10 μl). The matrix effect was constant in each matrix at low and high KET concentrations (100 and 5000 ng/ml) (Fig. 4).

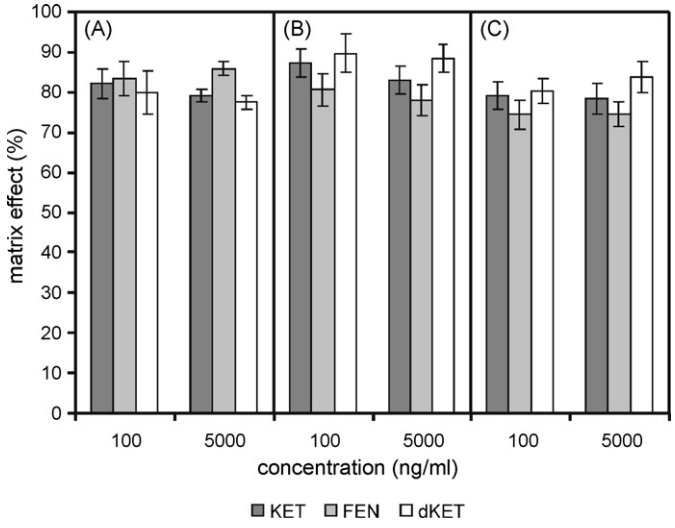

Fig. 4. Average matrix effect for (A) 1A, (B) MD-ISF and (C) OFM-ISF at two different KET concentrations (100 and 5000 ng/ml), $n = 3$ per matrix and concentration.

3.6. Linearity

Linear calibration curves ($y = ax + b$) were constructed using calibration standards from 0 to 10,000 ng/ml ketoprofen using the peak area ratio of ketoprofen to I.S. versus the nominal concentration. A $1/X$ weighting was used. Average calibration data, correlation coefficients and calculated LoDs obtained during method validation are given in Table 2. The Mandel test was passed for calibration curves up to 10,000 ng/ml obtained with either FEN or dKET as internal standards. Average correlation coefficients were >0.999 and calibration slopes did not alter significantly throughout the validation period which indicates a robust method.

3.7. Accuracy and precision

Accuracy and intra-batch and inter-batch precision data for the substitute matrix are presented in Table 3. The data calculated with dKET as internal standard are slightly more accurate compared to data calculated with FEN as the internal standard, particularly at concentrations near LoQ. This is consistent with the generally accepted opinion that isotopically labelled standards are better capable of balancing matrix effects than structural analogues because the matrix effect should not affect the relative efficiency of ionization of the drug and its isotope-labelled

Table 4
Accuracy data, % (%R.S.D.) for MD-ISF and OFM-ISF

Matrix	Conc KET (ng/ml)	Accuracy, % (%R.S.D.)[a]	
		FEN = IS	dKET = IS
MD-ISF	100	110 (6)	96 (2)
MD-ISF	5000	108 (4)	97 (2)
OFM-ISF	100	112 (6)	98 (6)
OFM-ISF	5000	112 (2)	98 (1)

[a] Obtained from Set A samples; $n = 3$ per matrix and concentration.

Table 5
Stability of samples after 7 days

I.S. used	Conc KET (ng/ml)	Accuracy, % (%R.S.D.)[a]				
		AT[b]	RT[c]	+4 °C	−80 °C	3 FTC[d]
FEN	200	101 (1)	108 (3)	109 (2)	107 (4)	109 (2)
FEN	5000	97 (0)	103 (3)	100 (1)	102 (3)	99 (4)
dKET	200	95 (1)	98 (5)	96 (1)	95 (1)	97 (3)
dKET	5000	92 (1)	94 (4)	91 (2)	91 (1)	91 (3)

[a] $n = 3$ per concentration and stability test.
[b] AT: after thawing.
[c] RT: room temperature.
[d] FTSC: freeze-thaw cycles.

I.S. [18]. However, both internal standards provided accuracy data that were well within the FDA criteria. In terms of intra-batch and inter-batch precision, both internal standards showed good precision (<6%) throughout the whole concentration range (Table 3). No difference between fenoprofen and the isotopically labelled ketoprofen could be observed in terms of precision.

Accuracy is a crucial issue and was thus additionally determined in dermal interstitial fluid obtained from rats, however, due to restricted availability this was performed with a minimum set of samples. The accuracy data for MD-ISF and OFM-ISF presented in Table 4 confirmed the data for both internal standards obtained with substitute matrix. As a consequence, both internal standards meet the requirements for reliable quantification given by the FDA.

3.8. Limit of quantification

A typical chromatogram of a 25 ng/ml QC control is shown in Fig. 3E. Signal-to-noise ratios were >1000 for ketoprofen peaks at 25 ng/ml and accuracy and precision data at 25 ng/ml (Table 3) were well within the FDA criteria for LoQ.

3.9. Stability of samples

Stability of ketoprofen could be assured for a storage period of seven days at −80, +4 °C and at room temperature as well as for three freeze-thaw cycles. No significant decrease could be observed (Table 5). Since stability of the sample could be demonstrated for storage at room temperature over a period of 7 days, long-term stability under freezing conditions (−80 °C) was considered not to be a critical issue and was therefore not assessed. In addition, long-term stability of ketoprofen in plasma has already been reported for 21 days at −20 °C [8] and for 5 months at −70 °C [13].

3.10. Application of the online-SPE-MS/MS method

The presented method was successfully applied to an OFM study in dermal tissue of rats. A representative time profile of ketoprofen concentrations in probe effluents after epicutaneous application of ketoprofen is shown in Fig. 5. The effluent concentrations peaked after 2–3 h to 5000 ng/ml and by the end of sampling decreased to approximately 500 ng/ml. The effluent concentrations varied considerably between catheters and between application areas within this test animal. Such variation may be explained by variation in probe insertion depth, in skin/stratum corneum thicknesses, in dermal capillary perfusion, and in natural local shunts through the barrier by hair follicles.

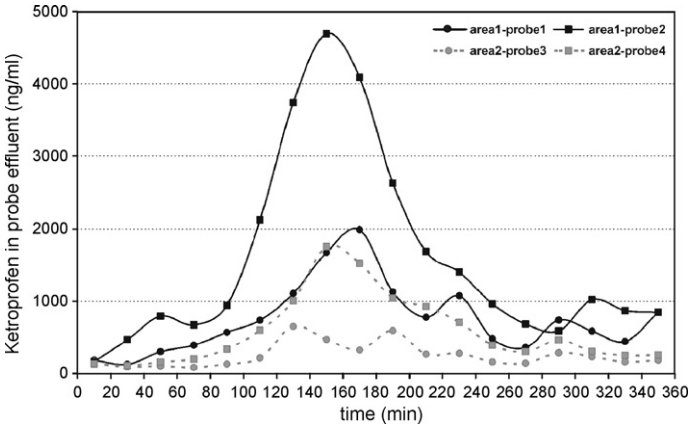

Fig. 5. Ketoprofen concentrations in the probe effluents from four dermal probes in rat no. 1.

4. Conclusion

We have presented a fast and easy to perform online-SPE-MS/MS method for the quantification of ketoprofen in ISF samples from microdialysis as well as open-flow microperfusion. Only a 10 μl sample volume is required for ketoprofen determination at levels from 25 to 5000 ng/ml. Sample processing is fast and efficient with high extraction recoveries of around 100%. A simple online-SPE-MS/MS setup with short run times (1.5 min) is used which enables complete analysis of one 96 well plate in duplicate injections in less than 5 h.

The method can either be performed using a commercially available and relatively cheap internal standard (fenoprofen) or an isotopically labelled internal standard (deuterated ketoprofen). Method validation showed that both internal standards meet the FDA criteria for reliable quantification within the tested concentration range. However, accuracy is improved with deuterated ketoprofen as the internal standard, particularly at ketoprofen levels near the limit of quantification. The method

was successfully applied to an open-flow microperfusion study performed in dermal tissue of rats.

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References

[1] D. Henry, L.L. Lim, L.A. Garcia Rodriguez, S. Perez Gutthann, J.L. Carson, M. Griffin, R. Savage, R. Logan, Y. Moride, C. Hawkey, S. Hill, J.T. Fries, BMJ 312 (1996) 1563.
[2] B. Mazieres, Drugs R.D. 6 (2005) 337.
[3] M. Kreilgaard, Adv. Drug Deliv. Rev. 54 (Suppl. 1) (2002) S99.
[4] E. Schnetz, M. Fartasch, Eur. J. Pharm. Sci. 12 (2001) 165.
[5] L. Schaupp, M. Ellmerer, G.A. Brunner, A. Wutte, G. Sendlhofer, Z. Trajanoski, F. Skrabal, T.R. Pieber, P. Wach, Am. J. Physiol. 276 (1999) E401.
[6] M. Bodenlenz, L.A. Schaupp, T. Druml, R. Sommer, A. Wutte, H.C. Schaller, F. Sinner, P. Wach, T.R. Pieber, Am. J. Physiol. Endocrinol. Metab. 289 (2005) E296.
[7] M. Ellmerer, L. Schaupp, G.A. Brunner, G. Sendlhofer, A. Wutte, P. Wach, T.R. Pieber, Am. J. Physiol. Endocrinol. Metab. 278 (2000) E352.
[8] V.V.P. Kumar, M.C.A. Vinu, A.V. Ramani, R. Mullangi, N.R. Srinivas, Biomed. Chromatogr. 20 (2006) 125.
[9] L. Montoya, L. Ambros, V. Kreil, R. Bonafine, G. Albarellos, R. Hallu, A. Soraci, Vet. Res. Commun. 28 (2004) 415.
[10] E. Yamamoto, K. Murata, Y. Ishihama, N. Asakawa, Anal. Sci. 17 (2001) 1155.
[11] H.J. Leis, M. Leis, W. Windischhofer, J. Mass Spectrom. 31 (1996) 486.
[12] S.H. Hoke, J.D. Pinkston, R.E. Bailey, S.L. Tanguay, T.H. Eichhold, Anal. Chem. 72 (2000) 4235.
[13] T.H. Eichhold, R.E. Bailey, S.L. Tanguay, S.H. Hoke, J. Mass Spectrom. 35 (2000) 504.
[14] I.R. Miksa, M.R. Cummings, R.H. Poppenga, J. Anal. Toxicol. 29 (2005) 95.
[15] H. He, X. Tang, F. Cui, Biol. Pharm. Bull. 29 (2006) 841.
[16] I. Tegeder, J. Lötsch, M. Kinzig-Schippers, F. Sörgel, G.R. Kelm, S.T. Meller, G. Geisslinger, Pharm. Res. 18 (2001) 980.
[17] Guidance for Industry Bioanalytical method validation, US Department of Health and Human Service, Food and Drug Administration, 2001.
[18] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[19] L. Bräutigam, G. Vetter, I. Tegeder, G. Heinkele, G. Geisslinger, J. Chromatogr. B Biomed. Sci. Appl. 761 (2001) 203.