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Rapid UPLC–MS/MS method for the determination of ketoprofen in human dermal microdialysis samples

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ABSTRACT

Dermal microdialysis (DMD) is a technique capable of determining the percutaneous penetration of drugs from topical formulations intended for local and/or regional activity. Typically, the concentrations of drug collected in dialysates are very low, generally in the ng/ml or even pg/ml range. An additional challenge is the very low volume of sample collected at each collection time and which can range from 1 to 30 μl only. Hence the objective was to develop and validate a rapid, accurate, precise, reproducible and highly sensitive LC–MS/MS method for the quantitative analysis of ketoprofen (KET) in dialystes following application of a topical gel product to the skin of human subjects. UPLC–MS/MS was used and KET was separated on an Acquity™ UPLC BEH C_{18} column (100 mm × 2.1 mm i.d., 1.7 μm) and analysed in negative-ion (NI) electrospray ionisation (ESI) mode. The mobile phase (MP) consisted of acetonitrile:methanol:water (60:20:20, v/v/v) under isocratic conditions at a flow rate of 0.3 ml/min. Samples were extracted using ethyl acetate with ibuprofen (IBU) as internal standard (IS) and the organic solvent was then evaporated to dryness and the residue re-constituted in methanol. 5 μl samples were injected and analysis was performed at ambient temperature 22 ± 0.5 °C. KET and IBU eluted at 1.07 and 1.49 min, respectively. KET and IBU responses were optimised at the transitions 253.00 > 209.00 and 205.00 > 161.00, respectively. Calibration curves were linear over the range 0.5–500 ng/ml with correlation coefficients > 0.999. The accuracy and precision of the method were found to be between 99.97% and 104.67% (R.S.D. < 2%) and the mean recovery of KET from normal saline was 88.03 ± 0.3% (R.S.D. < 2.20%). The LLOQ and LOD values were found to be 0.5 and 0.1 ng/ml respectively whereas the ULOD was set at 500 ng/ml. The method was successfully applied to determine the bioavailability of KET following application of topical KET gel, Fastum® gel, to the skin of human volunteers.

1. Introduction

Microdialysis (MD) is an *in vivo* technique involving the implantation of semi-permeable membrane systems into a specific region of tissue or fluid-filled space [1]. This technique is used for the continuous sampling of endogenous and/or exogenous compounds in extracellular spaces [2–4] which allows relative changes of concentration-time profiles of compounds to be described. This technique also mimics the functions of a capillary blood vessel which permits the exchange of solutes in and out of extracellular fluids (ECF) [3,5–7]. The principle of the MD technique is based on the passive diffusion of compounds down a concentration gradient across the semi-permeable membrane of a dialysis fibre [8] although facilitated diffusion has also been reported with the use of certain types of perfusates [9].

DMD, a relatively new application of MD, allows continuous monitoring of endogenous or exogenous solutes in the ECF with minimal tissue obliteration. The technique involves the placement of small perfused membrane systems at given depths within the dermis (Fig. 1). Dermal tissue is an ideal sampling site since the tissue is relatively uniform with the ECF in constant flux with the systemic circulation. Moreover, the implantation of the membrane system in the dermis involves a relatively simple procedure although training is imperative [8].

When a topical formulation is applied onto the skin and perfusate is pumped through an implanted membrane system, drug molecules from the topical formulation present in the dermal ECF diffuse into the membrane resulting in the presence of a net gain of drug in the perfusion medium collected (dialysate). The dialysate is collected at timed intervals and the drug concentration in the dialysate can be determined quantitatively.

A major limitation with the use of MD is the production of extremely small volumes (1–30 μl) of dialysate coupled with the extremely low drug concentrations (ng/ml or even pg/ml), which

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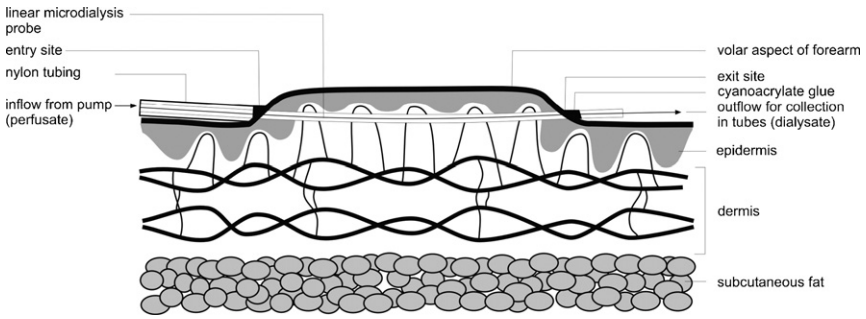

Fig. 1. Membrane system implanted into the dermis. Modified from Benfeldt [5].

requires a sensitive analytical method to measure the analyte [9]. Volumes deemed as sufficient will be dictated by the minimum volume required by the analytical instrumentation required for reproducible analysis. The generation of increased sample volume by increasing the perfusion rate, thereby decreasing the sample collection interval, results in sample dilution. Hence, an analytical method that either can make use of small sample volumes collected during the MD process, or having the necessary sensitivity to measure the drug concentration in the dialysate is essential [4]. Moreover, the temporal resolution is determined by a combination of perfusion rate through the MD probe and sample volume requirement of the analytical technique [10,11].

The recent introduction of ultra high performance liquid chromatography (UPLC) coupled to a mass spectrophotometer (MS) seeks to address the limitations associated with sensitivity and low volume samples. These systems possess high pressure pumps to accommodate the use of sub-2 μm particle size columns, with the sample injector system designed to handle fast injection cycles, low injection volumes, negligible carryover and temperature control (4–40 °C) which together contribute to rapid sample analysis [12]. Hence UPLC coupled to MS was chosen to provide for required fast, high-resolution separations having the necessary sensitivity and associated advantages over conventional LC–MS/MS systems.

Ketoprofen (KET) (Fig. 2), (*RS*)-2-(3-benzoylphenyl) propionic acid is an anionic non-steroidal anti-inflammatory drug (NSAID) and is widely used in the management and treatment of patients with rheumatic disease [13]. Topical administration of NSAIDs intended for local/regional activity offer the advantage of local and/or regional enhanced drug delivery to affected tissues, with reduced incidence of systemic adverse effects, such as peptic ulcer disease and gastrointestinal haemorrhage. The short distance of transmission from the site of application to the target site and the avoidance of the enterohepatic cycle are attractive benefits [14].

HPLC–MS/MS methods in NI ESI [15,16,18,19,21,22,24] or positive-ion (PI) ESI [23] or NI atmospheric pressure chemical ionisation (APCI) [17,20,25] for KET analysis have previously been reported. The main objective was to develop a rapid, accurate, precise, and reproducible UPLC–MS/MS method with the requisite sensitivity suitable for the quantitative determination of KET in extremely small volumes of DMD samples obtained from the topical application of KET gel products on human subjects.

2. Experimental

2.1. Reagents and chemicals

KET (99.4%), ibuprofen (IBU) (97%), naproxen (NAP) (98%) and flurbiprofen (FLU) (99%) were purchased from Sigma–Aldrich (Atlasville, South Africa). Acetonitrile 200 far UV ROMIL-SpS™ Super Purity Solvent and Methanol 215 ROMIL-SpS™ Super Purity Solvent were purchased from ROMIL Ltd. (Cambridge, UK). HPLC grade isopropyl alcohol was purchased from Burdick & Jackson, Inc. (Muskegon, MI, USA). The following analytical grade chemicals and reagents were obtained from Merck (Wadeville, South Africa); ethyl acetate, *n*-hexane, sodium dichromate dehydrate and sulphuric acid (98%, v/v). Sterile normal saline solution (sodium chloride 0.9%, m/v) was purchased from Bodene (Pty) Ltd. (Port Elizabeth, South Africa). HPLC grade water was obtained from a Milli-Q® A10 water purification system (Millipore, Molsheim, France) that comprised a Quantum™ EX ultrapure organex cartridge and a Q-Gard™ purification pack. The water was filtered through a 0.22 μm Millipak stack filter prior to use.

2.2. Instrumentation and equipment

Sample analysis was performed on an Acquity™ UPLC system (Waters® Corporation, Milford, MA, USA) which consisted of a binary pump solvent manager capable of generating pressures up to 1000 bar (~15 000 psi). Detection was carried out using an Acquity™ PDA UV–vis detector coupled in series with an Acquity™ TQD tandem-quadrupole MS equipped with a Z-spray electrospray interface (Manchester, UK). The UV–vis detector contained a 500-nl flow cell, the time constant was set at 25 ms and data sampled at

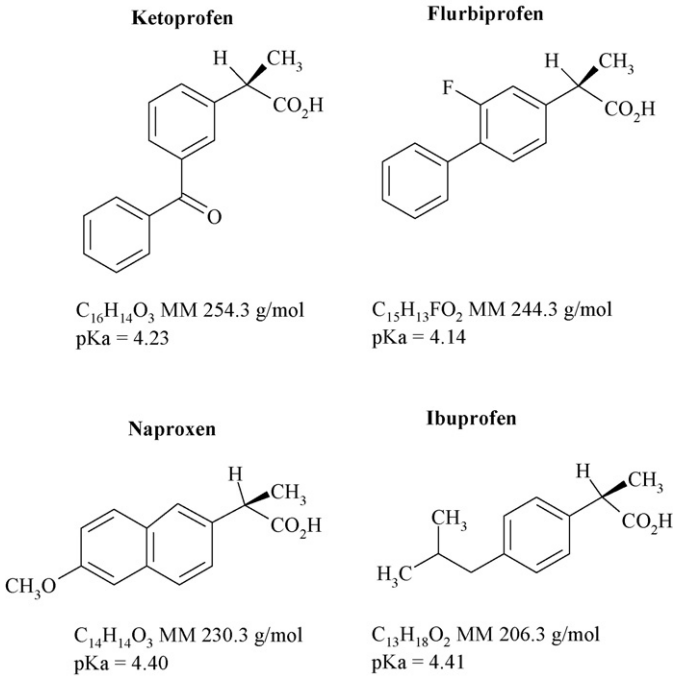

Fig. 2. Chemical structures of ketoprofen, flurbiprofen, naproxen and ibuprofen.

80 Hz. The system was equipped with strong and weak wash solution reservoirs. Instrument control, data acquisition and processing were carried out with MassLynx™ (version 4.1) and IntelliStart™ software (Waters® Corporation, Milford, MA, USA) was used to control the fluidics device to infuse solutions for tuning the MS. A Model MX5 analytical ultra-microbalance (Mettler Toledo, Greifensee, Zurich, Switzerland), a 200-μl pipette (Pipetman®, Gilson Medical Electronics, Villiers-le-Bel, France), a N-EVAP 24 place Model 112 nitrogen analytical evaporator (Organomation Associates Inc., South Berlin, MA, USA) coupled to a cylinder of high purity nitrogen gas (Afrox, Port Elizabeth, South Africa), an ultrasonic bath Model 8845-30 (Cole-Parmer Instruments, Chicago, IL, USA) and sample inserts (300 μl) (lot number 4170672080) with pre-installed plastic springs with amber (9 mm, screw top, 12 mm × 32 mm) sample vials from Waters® Corporation (Milford, MA, USA) were used for sample preparation.

2.3. Method development

2.3.1. UPLC conditions

An Acquity™ BEH C_{18} (100 mm × 2.1 mm i.d., 1.7 μm) stainless steel analytical column (Waters® Corporation, Milford, MA, USA) maintained at ambient temperature (22 ± 0.5 °C) was used for the analysis. 5 μl of samples were injected using the full loop option programmed with an overfill factor of 1.0. The needle displacement was set to 0.1 mm with both pre- and post-aspirate air gap maintained at automatic. Methanol/water and acetonitrile/water mixtures were initially investigated for use as mobile phase (MP). A MP consisting of acetonitrile/methanol/water (60:20:20, v/v/v) was used at flow rate of 0.30 ml/min and the eluate monitored at a UV wavelength of 255 nm. IBU, NAP and FLU were investigated as possible candidates for use as an internal standard (IS). The chemical structures of these compounds are shown above in Fig. 2.

2.3.2. MS tuning

The MS was tuned in NI ESI for the detection of precursor ions $[M-H]^-$ and the dissociation of these ions was induced. Methanolic solutions (100 ng/ml) of KET and IBU (IS) were infused at 10 μl/min under software control. Nitrogen, used as a nebulising and desolvation gas was provided by a high purity nitrogen generator NM 30LA 230VOC (Peak Scientific Instruments, Renfrewshire, Scotland, UK) and 2.55×10^{-4} mbar instrument grade argon (99.999%) (Afrox, Port Elizabeth, South Africa) was used as the collision gas.

2.3.3. MS optimisation

The most abundant ESI–MS/MS transition for each compound was monitored in the multiple reaction monitoring (MRM) mode to obtain the highest quantitative sensitivity. The choice of fragmentation products for each analyte based on the most intense signal and the optimisation of cone voltages, energy collisions and other instrument parameters were individually investigated for each compound in the combined flow-state mode through direct infusion of standard solutions in methanol (100 ng/ml). This was performed by UPLC where the MP was pumped directly into the MS via a switching valve. The optimised MS settings employed for both KET and IS were developed and maintained at the following: capillary voltage (3.50 kV), extractor voltage (2.00 V), RF lens voltage (0.50 V), source temperature (120 °C), desolvation temperature (500 °C), cone gas flow rate (109 l/h), desolvation gas flow rate (509 l/h), multiplier voltage (650 V) and gas cell Pirani pressure ($2.55e^{-3}$ mbar).

2.3.4. Sample carryover

Sample carryover was evaluated by sequential serial injections of a blank methanol solution, followed by the injection of a solution that contained a relatively high concentration (1 μg/ml) of KET and IS and subsequently by the injection of further blank solutions. Sample inserts and sample vials were also assessed for possible carryover or contamination. Approximately 30 μl of methanol was pipetted into randomly selected sample inserts from three separate lots (Lot number 4170672080), then placed in sample vials, capped and analysed.

2.3.5. Sample preparation and extraction

Calibration standards (0.5–500 μg/ml) and 3 quality control (QC) solutions of KET and IS (20 ng/ml) in normal saline solution were prepared. Equal volumes (30 μl) of KET and IS were pipetted into centrifuge tubes making a total volume of 60 μl. Samples were extracted using 200 μl of ethyl acetate and the tubes were vortexed for 10 s prior to centrifugation at 12,000 rpm (8050 × *g*) for 5 min. Approximately 180 μl of the supernatant from each centrifuge tube was pipetted into sample inserts which were placed into amber sample vials and evaporated to dryness in a nitrogen evaporator under a slow stream of high purity nitrogen gas. Samples were then reconstituted with 30 μl of methanol, the sample vials capped and vortexed for a further 10 s before analysis. Since KET photodegradation under normal laboratory lighting conditions at room temperature (22 ± 0.5 °C) has previously been established and reported [26,27], all sample handling was performed under filtered fluorescent (deep golden amber) light (Lee Filters, Andover, Hampshire, England). This precaution prevents the transmission of light at wavelengths lower than 530 nm and protects KET photodegradation. Amber glassware was used as a further precaution.

2.4. Method validation

2.4.1. Linearity

Plots of the ratios of the peak areas of KET/IS versus concentrations were plotted following the analysis of relevant standard solutions ($n = 3$) and the data were evaluated using linear regression analysis.

2.4.2. Accuracy and precision

Intra- and inter-day precision and accuracy of the assay were assessed over a period of 3 days using 3 QC standards (low, medium and high). Five separate determinations of each concentration were analysed to assess accuracy using working standards which were freshly prepared each day. For the precision studies, a single working stock solution was used and stored at ambient temperature under filtered light and also served as an indicator of stability.

2.4.3. Limits of quantification and detection

Standard stock solutions were diluted appropriately to obtain concentrations for the estimation of the limit of detection (LOD) and the lower limit of quantification (LLOQ) based on signal-to-noise (S/N) ratios of 3:1 and 10:1, respectively.

2.4.4. Recovery

Extraction recovery of KET was assessed by analysing spiked samples ($n = 5$) of three different concentrations corresponding to the concentrations of the QC samples (low, medium and high) and compared with data from the analysis of methanolic solutions of KET at similar concentrations.

2.5. Application of the UPLC–MS/MS method

The UPLC–MS/MS method was applied to analyse samples obtained from a DMD study to assess KET bioavailability from a topical gel formulation. Four MD probes were inserted into the dermis on the volar aspect of the forearms of 10 human subjects

and the probes were perfused (1.25 μl/min) with normal saline for 60 min. Two KET (2.5%, m/m) gel formulations, Fastum® gel (test) and Ketum® gel (reference), were applied (30 mg) to the skin (3 cm^2) directly overlying two probes each with samples collected at 30 min intervals for 5 h, stored in the dark at 4 °C and analysed within 24 h.

3. Results and discussion

3.1. Method development

3.1.1. UPLC conditions

To our knowledge, to-date there have not been any publications or reports which have focused on the development and validation of an analytical method using UPLC for the quantitative determination of KET for use in human DMD studies. Most published HPLC–MS/MS methods have reported the use of buffers or inclusion of acids or bases in the MP [15–17,20–23,25] for the analysis of KET, although there have been reports that used MPs without incorporating such reagents [18,19,24].

During the initial method development, the influence on retention time (RT), peak symmetry and detection sensitivity was investigated by varying the volume of the organic modifiers (methanol and/or acetonitrile) in the MP. Tailing of the leading edge of the KET peak was observed using a MP consisting of varying proportions of methanol/water. Sensitivity increased with increasing methanol content to 70% but was associated with peak broadening and deteriorating peak shapes. Sharp symmetrical peaks but with reduced sensitivity were observed when methanol was replaced with acetonitrile.

Initially, an isocratic MP consisting of acetonitrile/methanol/water (30:35:35, v/v/v) resulted in the requisite sensitivity, but the peaks were somewhat asymmetrical with tailing on the leading edge. As expected, increasing the composition of acetonitrile, while concurrently decreasing the composition of methanol (although maintaining an equal ratio of methanol/water), produced an acceptable symmetrical peak up to a ratio of 60:20:20, v/v/v (Fig. 3) without compromising sensitivity.

The associated increase or decrease in sensitivity with the use of methanol or acetonitrile respectively was in agreement with the previously indicated published methods/reports. The published methods that used an acetonitrile-based MP incorporated either acid (formic acid or acetic acid) or base modifiers (ammonium hydroxide) to achieve high sensitivity [15–17,20–23,25], whereas reports that used methanol/water MPs were not only unmodified (i.e., no acids or bases), but produced the highest sensitivity [18,19,24], which suggested that methanol might have a significant role in the ionisation of KET in NI ESI.

Another interesting observation reported in the published literature was the high sensitivity obtained with acid modifiers in NI ESI. It is commonly accepted that for weakly acidic analytes in NI mode, base modifiers facilitate analyte deprotonation resulting in increased sensitivity, whereas acid modifiers facilitate analyte protonation thereby resulting in decreased sensitivity. However, a number of weak carboxylic acids (e.g., acetic acid) have been reported to increase NI ESI response. The magnitude of this response however depends on the acid modifier, its concentration and the properties of the analyte [28]. The decreased sensitivity with acetonitrile/water MPs has been related to the unavailability of protons from either water or analyte for chemical reduction, which is the principal electrochemical reaction that occurs in NI ESI at the MS spray tip. The protons are reduced to hydrogen gas and thus additional protons provided by an acid modifier will facilitate the reduction process by providing excess negative charge hence increasing sensitivity [28]. Since methanol is a weak acid and may also make protons available to facilitate the reduction process, the increased sensitivity with methanol/water MPs may then be explained by the reduction potential of methanol.

The increased sensitivity observed with the combination of acetonitrile/methanol/water MP compositions confirmed that methanol indeed acted as an acid modifier, although increasing volumes of methanol resulted in asymmetric peaks. The decreased sensitivity observed when the methanol content was reduced by 5% or more, suggested that the volume of the acid modifier (methanol) insufficiently provided protons to the KET for chemical reduction. An isocratic composition of acetonitrile/methanol/water (60/20/20,

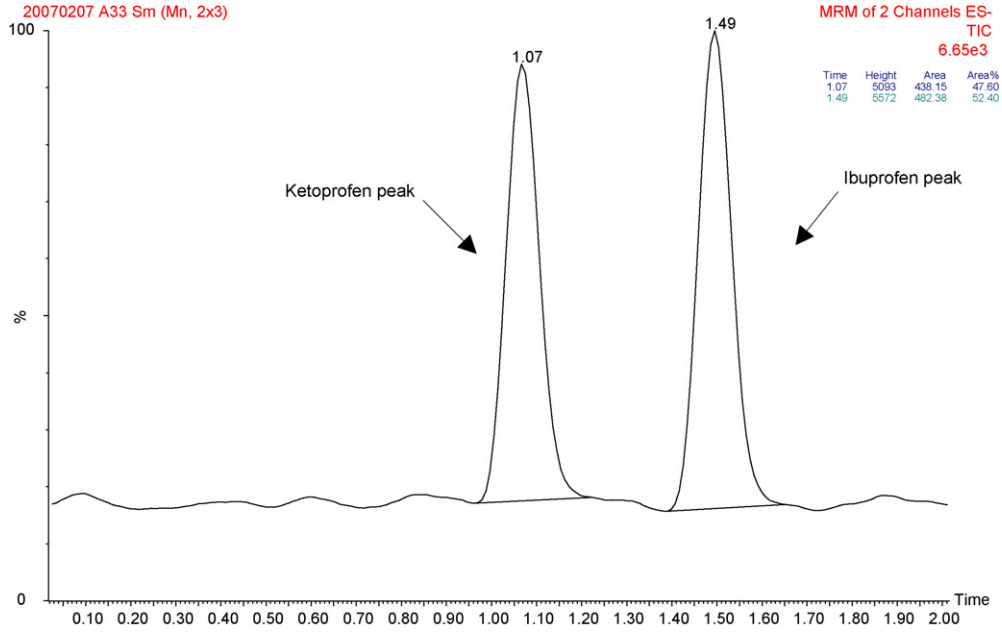

Fig. 3. ESI–MS/MS TIC chromatogram of KET (50 ng/ml) and IS (50 ng/ml). Conditions: Column: Acquity™ BEH C_{18} (100 mm × 2.1 mm i.d., 1.7 μm); Column temperature: 22 ± 0.5 °C; MP: (acetonitrile/methanol/water) (60:20:20, v/v/v); Flow rate: 0.3 ml/min; Injection volume: 5 μl; Ionisation mode: NI ESI; MRM: 253.00 > 209.00 and 205.00 > 161.00; Capillary voltage: 3.5 kV; Source temperature: 120 °C; Desolvation temperature: 500 °C; Desolvation gas flow: 509 l/h; Cone gas flow: 109 l/h.

Table 1
MRM NI ESI–MS/MS conditions for KET and IS.

Analyte	Collision (eV)	Cone voltage (V)	MRM transition (*m*/*z*)	Precursor and product ions (*m*/*z*)	
KET	8.0	15.0	253.00 > 209.00	252.94	$[M-H]^-$
				209.01	$[M-H-CO_2]^-$
				196.99	$[M-H-C_2H_3O_2]^-$
IBU	7.0	21.0	205.00 > 161.00	204.92	$[M-H]^-$
				160.94	$[M-H-CO_2]^-$
				158.99	$[M-H-CHO_2]^-$

v/v/v) at a flow rate of 0.30 ml/min was therefore deemed optimum for use as the MP.

No significant improvement in peak sensitivity was observed with increased flow rates, although shorter elution times were observed. Moreover the higher flow rates resulted in undesirable higher column back-pressures.

IBU, NAP and FLU, compounds which are structurally and physico-chemically similar to KET, were each investigated as an IS in order to compensate for possible errors in the extraction procedure. However, the latter two compounds co-eluted with KET at 1.21 and 1.15 min respectively, whereas IBU was well-resolved from KET and eluted at 1.49 min. The chosen flow rate of 0.3 ml/min was compatible with the use of IBU as IS (Fig. 3).

3.1.2. MS tuning and optimisation

NI ESI [15,16,18,19,21,22,24] was the commonly used ionisation mode presented in the published methods/reports. However, Daeseleire et al. [23] reported KET detection in PI ESI and conditions for NI APCI have also been reported [17,20,25]. The most sensitive methods/reports were attained with the use of NI ESI, hence this mode was subsequently investigated. The TQD MS tuned spectra of 100 ng/ml methanolic solutions of KET and IS revealed two product ions in each spectrum. Under the tuned conditions, product ions were produced for KET (*m*/*z* 209.01 and 196.99) and IBU (*m*/*z* 160.82 and 158.87) with the most abundant fragmentations observed were *m*/*z* 209.01 and *m*/*z* 160.82, respectively.

As previously mentioned, since the principal electrochemical reaction in NI ESI is reduction or deprotonation of molecules [M], the detection of the precursor ions was achieved by the loss of the carboxylic acid proton $[M-H]^-$. For both analytes, the product ions formed by the expulsion of CO_2 $[M-H-CO_2]^-$ were the most intense ions and this was in agreement with published MS/MS methods [15–19]. Secondary fragments $[M-H-C_2H_3O_2]^-$ and $[M-H-CO_2-H]^-$ corresponding to KET and IS respectively were too low to be used quantitatively. The MRM transitions used for the quantification of KET and IS are presented in Table 1.

3.1.3. Sample carryover

The UPLC system is equipped with two needle wash solution reservoirs, one containing a "weak" needle wash using 200 μl/run and the other a "strong" needle wash using 600 μl/run which clean the needle and wash station before, during and after each injection cycle. This is an added measure to avoid carry-over and trace contamination when using highly sensitive systems. During the initial stages of method development, small peaks were seen at the RTs of KET and IS following blank methanol injections. The composition of the strong wash solution was initially MP but subsequently replaced with (1500 μl/run) isopropanol alcohol/methanol/acetonitrile (40:35:35, v/v/v) while the weak wash solution (500 μl/run) was maintained with MP. This modification in the strong wash solution and change in the volumes of the washes resulted in the elimination of those peaks.

Different ESI–MS/MS responses using the same blank methanol solution from three lots of the same number (4170672080) of sample inserts were obtained. The analysis of lot 1 sample inserts showed no interference in both KET and IBU MRM transitions whereas blank injections from lot 2 inserts produced a response which interfered with the IBU MRM transition and blank injections from lot 3 inserts resulted in interference with both KET and IBU MRM transitions. Although the sample vials were only used as supports for the inserts, methanol was added to those vials and injected into the system but no response was observed indicating contamination only from lot 2 and 3 sample inserts.

Examination of the sample vials and inserts revealed that LCMS grade sample vials were supplied (Microsep, Sandton, South Africa) with HPLC grade sample inserts from the same supplier. Attempts were made to clean the sample inserts by individually flushing with methanol but without success. Further attempts were made to remove the contamination by immersing the inserts in a solution of chromic acid for 12 h followed by removal from the acid and thorough flushing, first with double distilled deionised water and then with HPLC grade water and finally sonicated in methanol for 30 min. This treatment was successful and all sample inserts were subsequently treated in this way prior to use.

3.2. Method validation

3.2.1. Linearity

The calibration plot for KET was linear with correlation coefficients better than 0.9999 ($y = 0.0427x + 0.0079$).

Table 2
Accuracy studies of KET DMD samples.

DMD samples	Day	Actual conc. (ng/ml)	Calculated conc. (ng/ml)	Accuracy (%)	Intra-day R.S.D. (%)
Low	1	1.46	1.52	104.11	3.22
	2	1.42	1.44	101.41	2.15
	3	1.48	1.49	100.67	2.51
Medium	1	152.32	152.61	100.19	1.52
	2	148.15	148.32	100.11	1.15
	3	154.41	154.43	100.01	1.05
High	1	453.84	453.71	99.97	0.57
	2	441.42	441.44	100.01	0.82
	3	460.07	460.10	100.01	0.25

Table 3
Precision studies of DMD samples.

DMD samples	Day 1 [mean conc. (ng/ml) ± S.D. (R.S.D.%) (*n* = 5)]	Day 2 [mean conc. (ng/ml) ± S.D. (R.S.D. %) (*n* = 5)]	Day 3 [mean conc. (ng/ml) ± S.D. (R.S.D. %) (*n* = 5)]	Inter-day [mean conc. (ng/ml) ± S.D. (R.S.D. %)]
Low	1.50 ± 0.02 (1.38)	1.52 ± 0.03 (1.96)	1.50 ± 0.02 (1.61)	1.51 ± 0.01 (0.67)
Medium	152.50 ± 0.28 (0.18)	152.62 ± 0.06 (0.04)	152.68 ± 0.03 (0.02)	152.60 ± 0.09 (0.06)
High	453.71 ± 0.01 (0.00)	453.71 ± 0.01 (0.00)	453.72 ± 0.01 (0.00)	453.71 ± 0.01 (0.00)

Table 4
Recovery studies of KET and IBU in DMD samples.

DMD samples	Actual conc. (ng/ml)	Calculated conc. (ng/ml)	% Recovery	% (*n* = 5)
Low	1.44	1.27	88.25	2.16
Medium	150.23	131.72	87.68	1.68
High	447.61	394.57	88.15	0.53
IBU	20.23	19.25	95.16	0.86

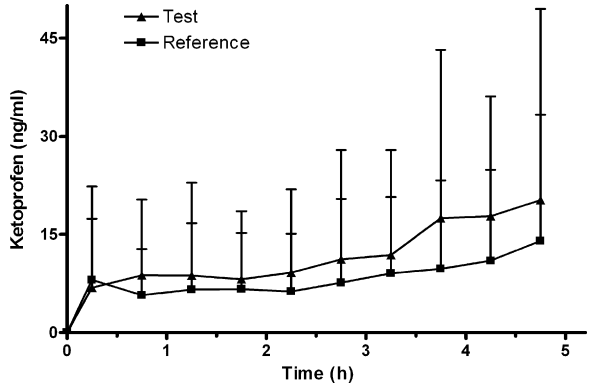

Fig. 4. Mean dialysate concentration-time profiles (±S.D.) (*n* = 10). Experimental: Dermal microdialysis technique, four probe insertions, four application sites, one probe per site, probes were 3 cm apart, probes covered approximately two quarters of the volar aspect of the forearm of each volunteer, 10 subjects, Formulations: Fastum® gel (Test) vs Ketum® gel (reference).

3.2.2. Accuracy and precision

The accuracy of the method was found to be in the range 99.97–104.67% with R.S.D.s less than 2% (Table 2). Data from the precision studies are presented in Table 3.

3.2.3. Limits of quantification and detection

The LOD and the LLOQ of KET were found to be 0.1 and 0.5 ng/ml respectively and the ULOQ set at 500 ng/ml.

3.2.4. Recovery

The recoveries of KET and IBU are depicted in Table 4.

3.3. Application of the UPLC–MS/MS method

The UPLC–MS/MS method was successfully employed to determine KET in DMD samples obtained from human subjects. A typical dialysate concentration-time profile is shown in Fig. 4.

4. Conclusions

An analytical method was developed and validated for the quantitative determination of KET in DMD samples. This method demonstrated that small injection sample volumes (5 μl) could be used to achieve rapid, accurate and reproducible analysis as well as provide the requisite sensitivity. Methanol acted as a weak acid which provided protons that promoted chemical reduction at the ESI spray tip thereby enhancing sensitivity. Optimisation of wash solution volumes was necessary which eliminated sample carryover and system contamination and sample inserts were identified as the source of interfering peaks which were subsequently cleaned with chromic acid. IBU was identified for use as IS which eluted at 1.49 min and the most abundant fragments in NI ESI for KET and IBU were 209.01 *m*/*z* and 160.82 *m*/*z* respectively. Normal saline solutions caused considerable signal suppression hence sample extraction was necessary. Calibration plots were linear over the range, 0.5–500 ng/ml and the method was accurate (99.97–104.67%) and precise with % R.S.D.s less than 2% and recovery of KET and IBU from DMD samples were approximately 88% and 95% respectively. The method was fully validated and successfully applied to assess KET bioavailability, from a topical formulation intended for local and/or regional activity, in a DMD study performed on human subjects.

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References

[1] C.S. Chaurasia, M. Müller, E.D. Bashaw, E. Benfeldt, J. Bolinder, R. Bullock, P.M. Bungay, E.C.M. de Lange, H. Derendorf, W.F. Elmquist, M. Hammarlund-Udenaes, C. Joukhadar, D.L. Kellog Jr., C.E. Lunte, C.H. Nordstrom, H. Rollema, R.J. Sawchuk, B.W.Y. Cheung, V.P. Shah, L. Stahle, U. Ungerstedt, D.F. Welty, H. Yeo, Pharm. Res. 24 (2007) 1014–1025.
[2] L. Groth, Cutaneous microdialysis: methodology and validation, PhD Thesis, Scandinavian University, 1996.
[3] L. Groth, P.G. Ortiz, E. Benfeldt, in: J. Serup, G.B.E. Jemec, G.L. Grove (Eds.), Handbook of Non-Invasive Methods and the Skin, thrid ed., CRC Press Taylor and Francis Group, FL, USA, 2006, pp. 443–454.
[4] N. Plock, C. Kloft, Eur. J. Pharm. Sci. 25 (2005) 1–24.
[5] E. Benfeldt, *In vivo* Microdialysis for the investigation of drug levels in the dermis and the effect of barrier perturbation on cutaneous drug penetration. Ph.D. Thesis, Scandinavian University, 1999.
[6] A. Peña, P. Liu, H. Derendorf, Adv. Drug Deliver. Rev. 45 (2000) 189–216.
[7] Y. Zhao, X. Liang, C.E. Lunte, Anal. Chim. Acta 316 (1995) 403–410.
[8] F. Mathy, A.R. Denet, B. Vroman, P. Clarys, A. Barel, R. Verbeeck, V. Préat, Skin Pharmacol. Appl. Skin Physiol. 16 (2003) 18–27.
[9] K.W. Ward, S.J. Medina, S.T. Portelli, K.M. Mahar Doan, M.D. Spengler, M.M. Ben, D. Lundberg, M.A. Levy, E.P. Chen, Biopharm. Drug Dispos. 24 (2003) 17–25.
[10] R. Verbeeck, Adv. Drug Deliver. Rev. 45 (2000) 217–228.
[11] F. Mathy, B. Vroman, D. Ntivunwa, A.J. de Winne, R. Verbeeck, V. Préat, J. Chromatogr. B 787 (2003) 323–331.
[12] L. Nováková, L. Matysová, P. Solich, Talanta 68 (2006) 908–918.
[13] C. Dollery, Therapeutic Drugs, vol. 2, Churchill Livingstone, London, UK, 1991.
[14] J.M.M. Evans, T.M. MacDonald, Drug Aging 9 (1996) 101–108.
[15] X. Miao, B.G. Koenig, C.D. Metcalfe, J. Chromatogr. A 952 (2002) 139–147.
[16] M. Farré, I. Ferrer, A. Ginebreda, M. Figueras, L. Olivella, L. Tirapu, M. Vilanova, D. Barceló, J. Chromatogr. A 938 (2001) 187–197.
[17] D. Löffler, T.A. Ternes, J. Chromatogr. A 1021 (2003) 133–144.
[18] M.D. Hernando, E. Heath, M. Petrovic, D. Barceló, Anal. Bioanal. Chem. 385 (2006) 985–991.
[19] S. Marchese, D. Perret, A. Gentili, R. Curini, F. Pastori, Chromatographia 58 (2003) 263–269.
[20] M.E. Abdel-Hamid, L. Novotny, H. Hamza, J. Pharmceut. Biomed. 24 (2001) 587–594.
[21] K.E. Pickl, C. Magnes, M. Bodenlenz, T.R. Pieber, F.M. Sinner, J. Chromatogr. B 850 (2007) 432–439.

[22] A. Panusa, G. Multari, G. Incarnato, L. Gagliardi, J. Pharmaceut. Biomed. Anal. 43 (2007) 1221–1227.
[23] E. Daeseleire, L. Mortier, H. de Ruyck, N. Geerts, Anal. Chim. Acta 488 (2003) 25–34.
[24] M. Gros, M. Petrović, D. Barceló, Talanta 70 (2006) 678–690.
[25] I. Tegeder, J. Lötsch, M. Kinzig-Schippers, F. Sörgel, G.R. Kelm, S.T. Meller, G. Geisslinger, Pharm. Res. 18 (2001) 980–986.
[26] R.N.O. Tettey-Amlalo, *In vitro* release of Ketoprofen from proprietary and extemporaneously manufactured gels, MSc Thesis, Rhodes University, Grahamstown South Africa, 2005.
[27] D.K. Bempong, L. Bhattacharyya, J. Chromatogr. A 1073 (2005) 341–346.
[28] Z. Wu, W. Gao, M.A. Phelps, D. Wu, D.D. Miller, J.T. Dalton, Anal. Chem. 76 (2004) 839–847.