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Determination of two COX-2 inhibitors in serum and synovial fluid of patients with inflammatory arthritis by ultra performance liquid chromatography–inductively coupled plasma mass spectroscopy and quadrupole time-of-flight mass spectrometry

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ABSTRACT

The determination of two sulphur-containing drugs, the COX-2 inhibitors celecoxib and etoricoxib, in the serum and synovial fluid of inflammatory arthritis patients, is described using a sensitive ultra performance liquid chromatography–inductively coupled plasma mass spectroscopy (UPLC/ICPMS) method. Confirmation of the identity of the analytes in the samples was also performed by electrospray quadruple time-of-flight mass spectrometry in positive electrospray ionisation mode. The two COX-2 inhibitors were extracted from serum and synovial fluid following dilution with acetate buffer (pH 5) and liquid–liquid extraction (LLE) into ethyl acetate. Extracted samples were then analysed using UPLC/ICPMS with sulphur-specific detection. The limit of detection by UPLC/ICPMS was 0.45 ng/ml of sulphur in both serum and synovial fluid. The UPLC/ICPMS method was applied to the analysis of samples from patients receiving either 200 mg/day of celecoxib (2× 100 mg), 90 mg/day etoricoxib or placebo. The range of concentrations detected in the samples for the two drugs was from 0.3 to 3.3 μg/ml.

1. Introduction

COX (cyclooxygenase) enzymes are responsible for the formation of important biological mediators; such as prostaglandins in response to an inflammatory insult [1]. Pharmacological inhibition of COX can provide relief from the symptoms of inflammation and pain and many COX inhibitors have been introduced as analgesics and non-steroidal anti-inflammatory drugs (NSAIDs). Conventional NSAIDs inhibit both forms of COX. The assumption that the anti-inflammatory efficacy is due to inhibition of COX-2 whilst toxic side-effects are mainly caused by inhibition of COX-1 has led to the development of selective COX-2 inhibitors [2].

Celecoxib (CXB) (IUPAC 4-[5-(4-methylphenyl)-3-(trifluoromethyl) pyrazol-1-yl]benzenesulphonamide) (Fig. 1) was the first selective COX-2 inhibitor developed to reduce the serious side-effects of NSAIDs associated with the inhibition of COX-1 seen with non-selective COX inhibitors. It was introduced to clinical practice in 1999 and it is marketed under the brand names Celebrex. Its bioavailability is ca. 40% following oral administration with protein binding of 97% (mainly to serum albumin). Its metabolism is hepatic, primarily via CYP2C9 and the drug has a plasma half-life of ca. 11 h. [3] A number of analytical methods have been reported for CXB reviewed by Rao et al. [4] and LC–MS methods for the quantitation of CXB in human plasma have been described [5,6].

Etoricoxib (EXB) (IUPAC 5-chloro-2-(6-methylpyridin-3-yl)-3-(4-methylsulphonylphenyl)pyridine) (Fig. 1), commercially available under the brand names: Arcoxia, Nucoxia, Tauxib and Algix is a more recent COX-2 selective inhibitor, introduced for the treatment of osteoarthritis [7–9]. Current therapeutic indications for EXB in addition to osteoarthritis include: treatment of rheumatoid

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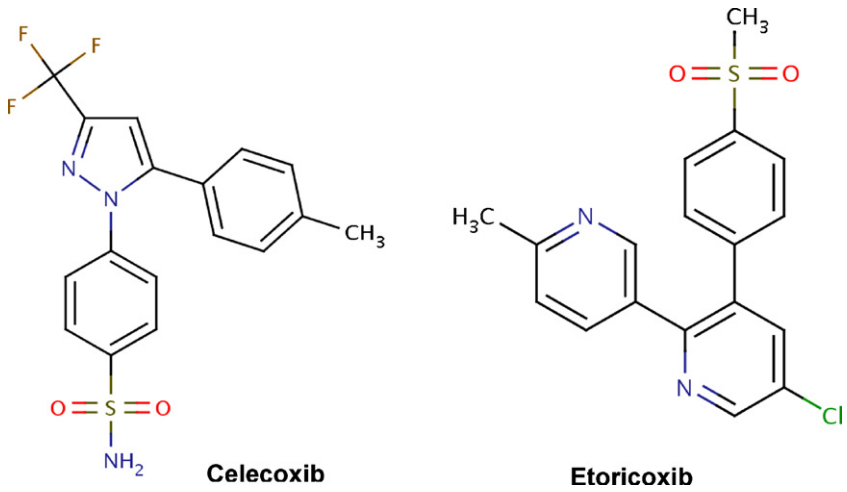

Fig. 1. Chemical structure of the two COX-2 inhibitors.

arthritis, ankylosing spondylitis, chronic low back pain, acute pain and gout. As a second-generation COX-2 inhibitor it has higher *in vitro* selectivity compared to other drugs marketed currently. Its bioavailability is 100% following oral administration with a protein binding of 92%. The biotransformation of EXB is hepatic, primarily via CYP3A4 and the drug has a plasma half-life of ca. 22 h [10,11]. As a result of its relatively recent introduction very few analytical methods have so far been reported for EXB (see Ref. [4]). Of the published methods for EXB three are based on LC–MS [12–14].

Whilst LC/MS methods are increasingly becoming the standard means of obtaining sensitive and specific bioanalytical methodologies for drugs and their metabolites in biological fluids, other strategies can be used to achieve these aims. Thus drug substances containing heteroatoms can also be readily detected using element-specific detectors such as inductively coupled plasma mass spectrometry (ICPMS). ICPMS has been widely used as an element-selective detector for both gas and liquid chromatography [15,16] and it is particularly suited for coupling with liquid chromatography, because of its compatibility with typical LC flow rates. It offers accurate and low detection limits of elements as the ICP is an excellent ion generator (under typical plasma conditions only a few elements are less than 90% ionised). ICPMS has recently found many bioanalytical applications [17–22] and element-specific detection via quadrupole ICPMS of sulphur-containing drugs has been successfully applied [23,24].

In this study the specific detection of the two sulphur-containing drug substances EXB and CXB by ICPMS in serum and synovial fluid samples was performed. Chromatographic separation was performed by UPLC (ultra performance liquid chromatography) with the aim of further increasing the sensitivity of the method. To date relatively few applications of UPLC coupled with ICPMS have been reported, but these include speciation analysis of bromine containing preservatives and selenium in urine [25,26]. To the best of our knowledge this is the first report of the application of UPLC and ICPMS technologies for the analysis of pharmaceuticals in either serum or synovial fluid.

2. Experimental

2.1. Materials

All solvents used for chromatography and ethyl acetate for liquid extraction were of HPLC grade and obtained from Fisher Scientific (Loughborough, Leicestershire, UK). Formic acid, ammonium acetate and glacial acetic acid of analytical grade were also purchased from Fisher Scientific. Water (18.2 MΩ) was obtained from a Purelab Ultra system from Elga (Bucks, UK).

2.2. Instrumentation

A Waters® ACQUITY UPLC System (Ultra performance Liquid chromatograph) coupled either with a Perkin-Elmer ELAN® DRC II ICPMS (Beaconsfield, UK) or with a Waters MicroMass Q-TOF Micro (Milford, MA, USA) mass spectrometer was used for the data acquisition.

2.3. Sample collection

Serum and synovial fluid samples were collected from 51 patients of over 18 years of age. All the subjects suffered from inflammatory arthritis and were diagnosed with a swollen major joint and accumulated liquid (mainly in the knee). The sample collection was performed in two visits. In the first visit serum and synovial fluid were collected from all 51 patients after clinical examination and before any treatment. After that they were separated into three groups. The first group ($n = 17$) was administered with CXB (Celebrex) 100 mg twice a day for 5 days, the second ($n = 17$) with EXB (Arcoxia) 90 mg once per day for 5 days and the third ($n = 17$) did not take any anti-inflammatory drug for the duration of the study. In the second visit serum and synovial fluid were collected after the drug ingestion (3 h in the case of Celebrex and 1 h in the case of Arcoxia), at the time of the theoretical Cmax in blood [27–29]. The samples were then stored at −80 °C until extracted for analysis.

All individuals volunteered for the study, read and agreed with the experimental protocol and signed the written consent form. Aspiration of the synovial fluid is a common and necessary practice in the therapeutic intervention of such patients. All samples originated from programmed visits of patients in the rheumatology unit of AHEPA University hospital and were collected by a MD rheumatologist. The study had the approval of the AHEPA hospital scientific committee and the research committee of Aristotle University.

2.4. Sample treatment

The sample treatment method used was adapted from reference [6] with slight modifications. The method was selected as it is reported to give satisfactory mean recovery percentages for celecoxib (>90%) at three concentration levels in plasma. Here an aliquot

of 200 μl of each sample (serum or synovial fluid) was mixed with 200 μl of 0.1 M acetate buffer, pH 5. The mixture was then extracted with 960 μl of ethyl acetate by vortexing vigorously for 10 min. After the mixture was centrifuged at 10,000 rpm for 13 min the vials were kept at −20 °C. After the lower aqueous phase was frozen the upper organic layer was decanted into a 96-well plate. Evaporation to dryness under a stream of nitrogen at 30 °C was achieved with the aid of a miniVap sample concentrator for 96-well plates (Porvair sciences). Reconstitution of the residue with 200 μl of methanol was then performed and a 10-μl aliquot of this sample was injected into the UPLC system.

Stock and working solutions were prepared from the standard compounds. For the construction of the calibration curves for the two sample types, pooled drug-free serum or synovial fluid samples were spiked with the two drugs. For this aliquots of 100 μl were mixed with 100 μl of standard mixtures of EXB and CXB at a concentration range of 0.95–20.00 μg/ml so that the final concentration of the spiked samples were from 0.48 to 10.00 μg/ml. These samples were then treated in the same way as described above in order to construct calibration curves. The samples were injected in triplicate and the resulting peak areas were averaged for the calculation of the standard curve.

2.5. Sample analysis

2.5.1. UPLC conditions

An Acquity C18 BEH (1.7 μm 2.1 mm × 150 mm) column was used at 50 °C with a binary solvent system of A: water 0.1% formic acid and B: methanol 0.1% formic acid. A gradient elution program starting at 10% B (v/v) and changing to 100% B (v/v) over 7 min was applied. The flow rate was set at 0.3 ml/min. Re-equilibration at 10% B (v/v) for 3 min was applied prior to the next injection (injection volume was 10 μl).

2.5.2. UPLC/ICPMS

ICPMS was carried out on an ELAN® DRC II ICPMS (PerkinElmer, Beaconsfield, UK). The operating acquisition conditions were set as follows: Nebulizer gas flow: 1.3 l/min, ICP RF power: 1500 W, lens voltage: 5 V, analog stage voltage: −1575 V, pulse stage voltage 750 V. Scan mode: peak hopping, dwell time (per amu): 150 ms, cell gas A: 0, cell gas B: 0.6, RP(q): 0.5, mass monitored: 47.967, acquisition mode: DRC (dynamic reaction cell). The DRC is normally used to remove plasma-based interferences by making use of reaction gases such as methane and ammonia, which are infused into the DRC, which then react with the isobaric molecules. However, in this case a small amount of oxygen was added to the DRC in place of the reaction gases to obtain a "chemical resolution" effect in which sulphur and oxygen react to produce sulphur oxide species. This species provides a 100-fold increase in sensitivity over the sulphur element alone and is detected in a region free from isobaric interferences [30–34]. The sample introduction system consisted of a cross flow nebulizer and a Peltier cooled cyclonic spray chamber. The nebulizer converts the eluent from the UPLC into fine droplets, which are carried through to the Peltier spray chamber using argon carrier gas. The Peltier temperature was held at −5 °C condensing out the organic portion and large droplets of the mobile phase with the fine droplets passing into the centre channel of the torch and then into the plasma.

2.5.3. UPLC/TOF MS/MS

The TOF data were acquired on a Waters Micromass Q-TOF Micro time-of-flight (TOF) mass spectrometer using positive electrospray ionisation (ESI). Nitrogen was used as the nebulizing and desolvation gas, with the source operated at 150 °C. The capillary voltage was set at 3500 V, the desolvation temperature at 300 °C, the cone voltage at 35 V, the cone gas at 10 l/h and the desolvation gas at 1000 l/h. Argon was used as the collision gas.

MS data over the range 100–600 amu were acquired together with MS/MS data in a single run: for EXB the fragment ion m/z 280.5 of the MH^+ m/z 359.5 was monitored from 3 to 4 min at the mass scan range 100–350 amu. For CXB the fragment ion at m/z 282.6 of the parent ion MH^+ m/z 382 was monitored from 5 to 7 min at the mass scan range 100–380 amu. Ion energy and collision energy for both drugs were set at 4.3 and 30 V, respectively.

The scan time was set at 0.5 s with an interscan delay at 0.1 s. A lock mass of leucine enkephalin (556.2771 amu) at a concentration of 25 fmol/μl was employed at a flow rate of 2 μg/ml via a lockspray interface. At the same time data were collected with a DAD giving a summed trace from 190 to 400 nm.

3. Results and discussion

Since the two drug substances both contain one sulphur atom (Fig. 1), an element-specific detection and analysis method such as UPLC/ICPMS should provide excellent selectivity. However, a complication of this approach is that sulphur detection by ICPMS is compromised by a range of interfering polyatomic ions and this often leads to high backgrounds and poor sensitivity. Thus main isotope of sulphur (^{32}S, abundance 95.018%) suffers from a serious interference as a result of the polyatomic ion $^{16}O^{16}O^+$ at nominal m/z 32 and to avoid this in the current application the use of dynamic reaction cell technology was employed. The DRC can be used to eliminate isobaric interferences [30–34] and thus provides increased sensitivity for S detection. In DRC mode a chamber placed before the traditional quadrupole space of an ICPMS device allows the reaction of sulphur (S^+) with oxygen O_2 to form sulphoxide (SO^+) and thereby move the detection from m/z 32 to m/z 48, a region that suffers from much less interference. The use of oxygen as reaction gas in the collision cell in an ICPMS method in order to increase the detection sensitivity for compounds containing S element has been reported previously [33]. An increase in response for S of 100 times for the SO^+ compared to that of the ^{34}S isotope, and 1000 times compared to ^{32}S, was shown. Thus, such an application of LC/ICPMS is ideal for the analysis of compounds containing S at low concentrations in complex matrices such as biological fluids. The sensitive detection of drug substances containing S in biological samples such as urine have been demonstrated previously [34] for the profiling of drug metabolites in urine.

As well as using the DRC to further increase sensitivity the ICPMS was coupled with an UPLC system. As a result of employing sub 2 μm particles for the stationary phase at conventional flow rates UPLC provides faster analysis, high-resolution separations and high sensitivity due to more efficient chromatography [35]. In Fig. 2 an ICPMS chromatogram of serum samples spiked with (10 μg/ml) EXB and (3.5 μg/ml) CXB monitored at m/z 48 is shown.

3.1. UPLC/ICPMS method validation

The peak area ratio of CXB and EXB was used to construct calibration curves by weighted linear regression of standard CXB and EXB concentration versus measured peak area ratio. Concentrations in unknown serum and synovial samples were determined by interpolation from the calibration curve. The linear dynamic range was from 0.48 to 10 μg/ml with a R^2 value of 0.996 and 0.991 for EXB and CXB, respectively. The limit of detection (LOD) was assessed as the concentration giving a signal of three times the noise signal (3S/N) whilst in this instance the limit of quantification was taken as 10 time the S/N. The LOD was found to be 0.55 ng (corresponding to 4.5 pg of sulphur) on column for EXB and CXB.

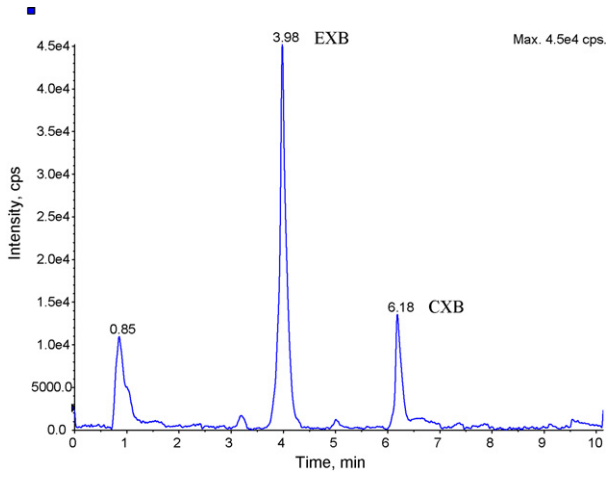

Fig. 2. ICPMS chromatogram of serum sample spiked with 10 μg/ml etoricoxib (3.98 min) and 3.5 μg/ml celecoxib (6.18 min).

The intraday accuracy and precision were determined by replicate ($n = 5$) analysis of standards at two concentration levels (medium and low) within the linear range. The accuracy reported in terms of percentage relative error, ranged from 94% to 105% for EXB and from 95% to 105% for CXB.

The precision in terms of relative standard deviation (R.S.D.) as assessed by replicate analysis of standards was found to be 3.4% and 2.8%, respectively for EXB and CXB.

3.2. Application to patient samples

The currently reported assay was developed to support clinical studies in these patients on drug penetration and its correlation to the level of cytokines and other marker molecules.

CXB was detected in the serum of all 17 patients to which the drug had been administered, in concentrations ranging from 0.35 to 1.85 μg/ml. However, CXB was found in synovial fluid samples only in 6 cases, with concentrations between 0.33 and 0.79 μg/ml. EXB was also found in the serum of all 17 patients administered with the drug, in concentrations ranging from 1.0 to 3.3 μg/ml and in contrast to CXB also in all 17 of the synovial fluid samples in concentrations ranging between 0.49 and 2.29 μg/ml. In Table 1 the concentrations of the two COX-2 inhibitors found in the samples from each of the subjects are given.

In Figs. 3 and 4 ICPMS typical chromatograms of serum (a) and synovial fluid (b) from patients before and after administration with EXB and CXB are given. The peak of EXB had a retention time of 3.9 min whereas the peak of CXB was eluted at 6.1 min. In Fig. 3a and b the peaks correspond to 1 μg/ml CXB in serum and 0.7 μg/ml in synovial fluid respectively whereas in Fig. 4a and b the concentrations correspond to 2.3 μg/ml EXB in serum and 2.4 μg/ml in synovial fluid respectively. Whilst a number of endogenous sulphur-containing metabolites were seen in blank pre-dose samples no interferences were detected eluting at the retention times of the analytes using either ICPMS or Q-TOF in either serum or synovial fluid. The Q-TOF MS/MS functionality was used to provide unambiguous confirmation of the presence of the drug molecules in samples obtained from dosed patients, thereby confirming the specificity of the ICPMS. Additionally, in the samples obtained from the patients treated with placebo no drug was detected in either sample type, using both ICPMS and Q-TOF detectors.

As indicated above, the presence of the two drugs in the samples was unambiguously confirmed by analysis via the Q-TOF in MS/MS mode. The MS spectra shown in Fig. 5 are those of the two compounds on which the confirmation of the peaks was based. In the case of EXB the chlorine isotope pattern is obvious, where the molecular ion peak MH^+ at m/z 359.5 appears together with a MH^{2+} peak at m/z 361.5 in a height ratio 3:1 corresponding to the presence of the two chlorine isotopes, ^{35}Cl and ^{37}Cl in the molecule. This diagnostic isotope pattern is also clear in the fragment ion m/z 280.5 containing chlorine.

The above data given in Table 1 are of much interest in terms of the distribution of the two COX-2 inhibitors into the region of inflammation, revealing EXB to have a higher exposure in the synovial fluid compared to CXB. It is also clear that, at the time point measured EXB is generally present at higher concentration in serum samples than CXB. This result is in agreement with the reported higher bioavailablity of EXB [36,3]. Concentrations of both drugs were found to be variable in synovial fluid which could be related to the varying extent of inflammation of the synovial membrane (joint-lining) in each patient and the amount and condition of the accumulated liquid.

3.3. Advantages of the method

One of the features of using the ICPMS for this type of work is that it acts as a general detector for sulphur, showing both drug and endogenous sulphur-containing compounds, with, in addition, the potential to detect compound-related material such a metabolites.

Table 1
Concentrations of celecoxib and etoricoxib in serum and synovial fluid of patients with inflammatory arthritis administered with one or the other COX-2 inhibitor.

Celecoxib			Etoricoxib		
Sample number	Serum concentration (μg/ml)	Synovial fluid concentration (μg/ml)	Sample number	Serum concentration (μg/ml)	Synovial fluid concentration (μg/ml)
# 1	0.540	0.000	# 5	1.111	0.494
# 2	1.300	0.000	# 6	1.855	0.871
# 3	1.855	0.000	# 7	2.652	0.676
# 4	1.369	0.000	# 10	2.634	1.255
# 8	0.346	0.000	# 14	2.034	0.965
# 9	0.4607	0.000	# 15	2.111	0.673
# 11	0.538	0.344	# 16	3.301	0.991
# 12	0.999	0.724	# 18	2.335	1.743
# 13	0.524	0.344	# 19	1.772	1.055
# 17	0.591	0.000	# 20	1.023	0.565
# 21	0.753	0.473	# 22	3.060	1.359
# 25	0.609	0.333	# 23	2.060	0.652
# 27	1.028	0.789	# 24	2.332	2.292
# 30	0.379	0.000	# 26	2.868	0.929
# 31	0.575	0.000	# 28	1.623	1.449
# 32	0.569	0.000	# 29	2.252	0.636
# 34	0.393	0.000	# 33	2.154	1.161

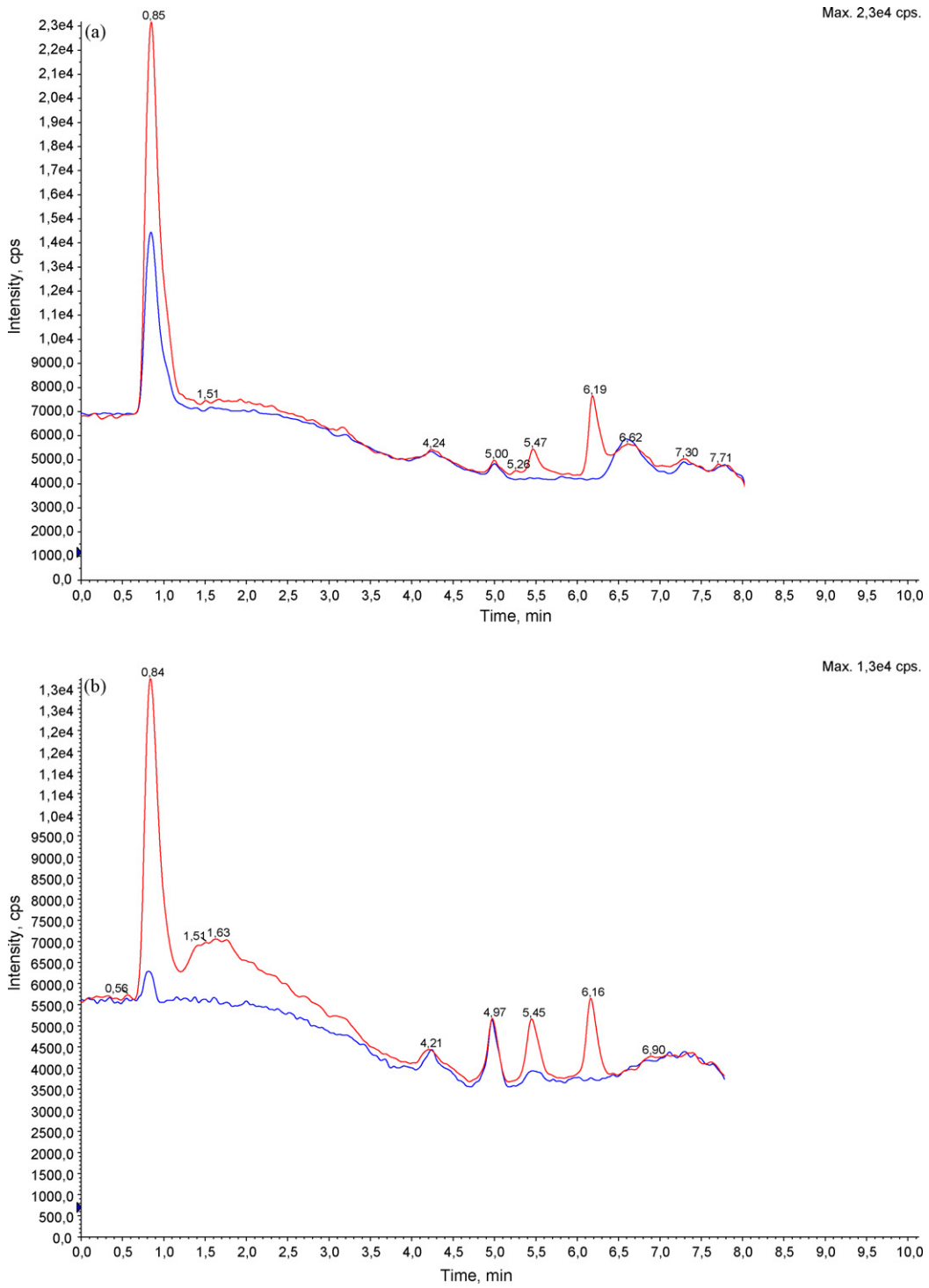

Fig. 3. ICPMS chromatogram of (a) serum sample from patient before (blue trace) and after administration of celecoxib 1 μg/ml (red trace) and (b) synovial fluid from the same patient before (blue trace) and after administration of celecoxib 0.7 μg/ml (red trace). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Examination of the post-dose traces of serum and synovial fluids samples of CXB revealed a sulphur-containing peak eluting at 5.4 min, slightly earlier than the drug itself in both serum and synovial fluid samples (in Fig. 3 the unknown peak is shown at 5.4 min in both serum and synovial fluid samples in post-dose sample). However, the Q-TOF mass spectral data obtained for this peak (having the most abundant spectral peak at m/z 358.7) was unrevealing and the unknown did not seem to correspond to any of the known metabolites of CXB (both two COX II inhibitors are metabolised to the primary alcohol and the corresponding carboxylic acid with nominal molecular masses of 397 and 411 respectively) [37,38].

In the case of EXB two small sulphur-containing peaks were seen eluting shortly after the main peak eluting (0.17 and 0.25 min later) in all post-dose samples (Fig. 4). However, as with CXB examination of the Q-TOF mass spectra for these peaks, particularly the absence of a chlorine isotope pattern in the presumed molecular ions, suggested that they were unrelated to the drug, and more likely endogenous compounds. Clearly further work, outside the

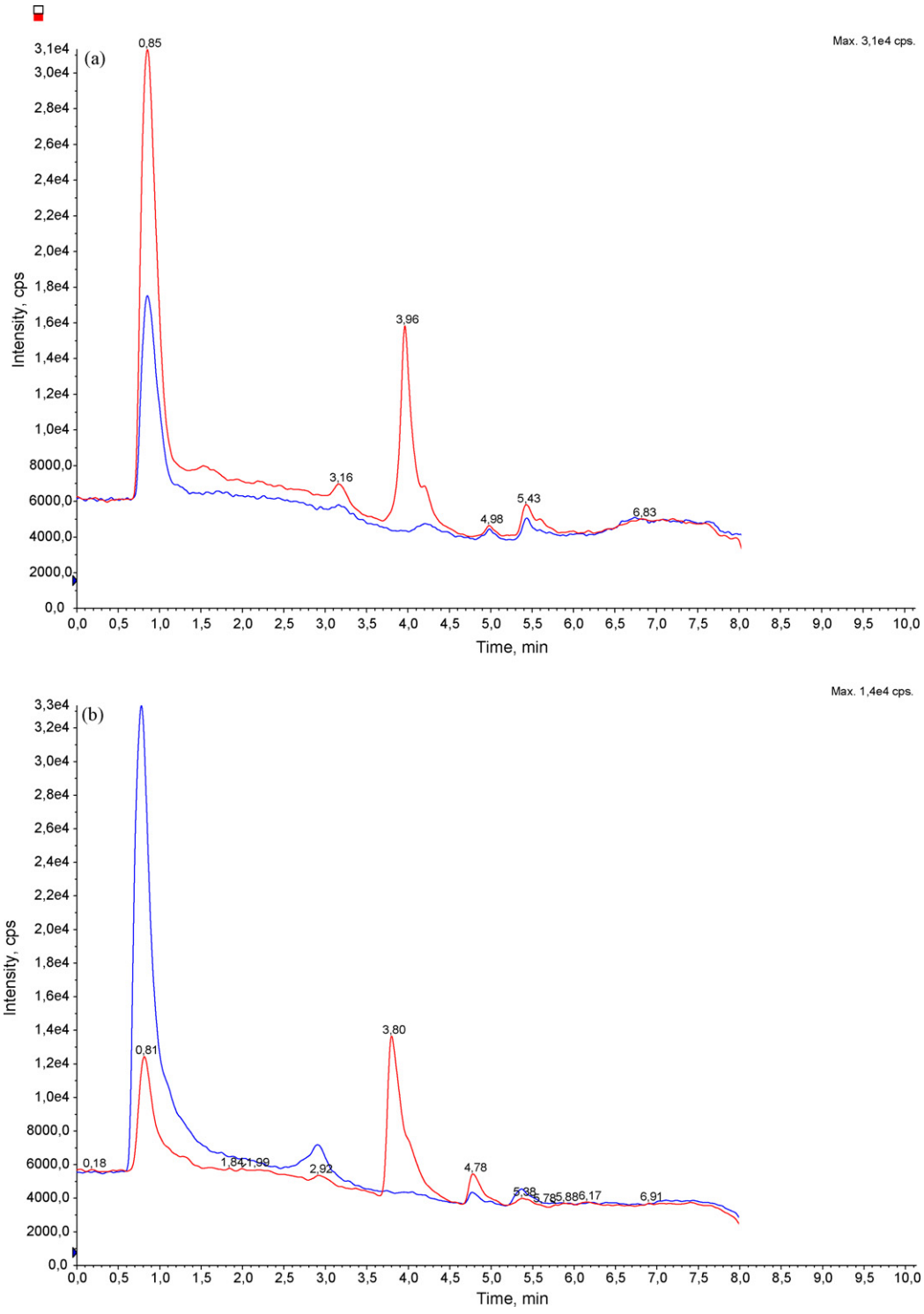

Fig. 4. ICPMS chromatograms of (a) serum sample from patient before (blue trace) and after administration of etoricoxib 2.3 μg/ml (red trace) and (b) synovial fluid sample from the same patient before (blue trace) and after administration of etoricoxib 2.4 μg/ml (red trace). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

scope of the present study, would be required to characterise these substances.

As mentioned above the LOD in UPLC/ICPMS analysis was 0.55 ng on column for EXB and CXB. These values are similar to those obtained for the UPLC/Q-TOF MS/MS where the LOD of detection for EXB and CXB were 0.10 and 0.12 ng (on column) respectively. Despite the fact that the Q-TOF MS/MS could provide marginally lower detection limits the ICPMS was used for quantification purposes since it provides a wide linear range and the LOD was more than adequate for the needs of this study. It is also likely that, with further optimisation to reduce extra-column band broadening the LOD of UPLC/ICPMS in this application could be improved further. Some evidence for extra-column peak broadening can be seen in the peak base widths. In the UPLC/ICPMS peak base widths were found to be 20–24 s whilst in the UPLC/Q-TOF MS/MS chromatograms these were between 15 and 20 s. UPLC provides superior

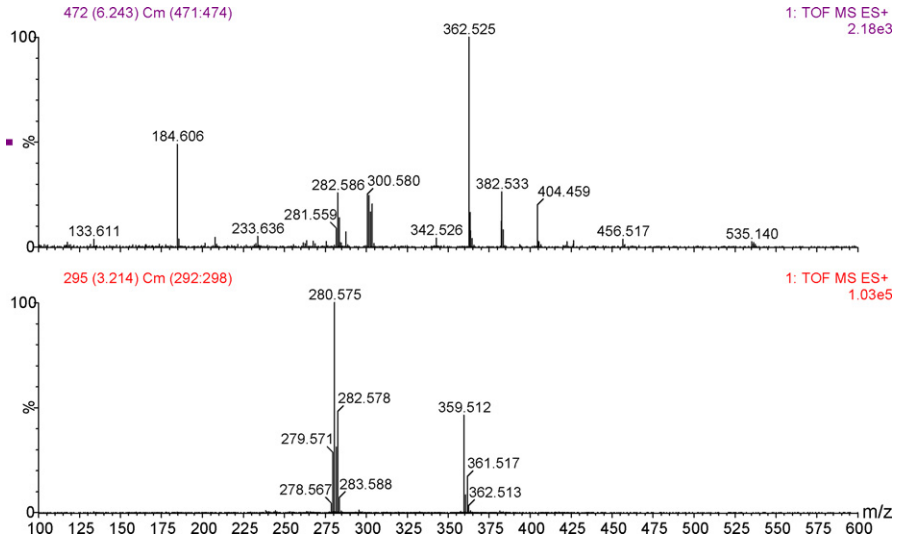

Fig. 5. (+ESI) Q-TOF spectrum of celecoxib (upper) and etoricoxib (below).

chromatographic efficiency due to utilisation of sub 2 μm particles which are combined with advanced fluidics employing narrow peak tubing and minimal flow paths in order to suppress peak broadening effects. Some of these advantages are compromised when the UPLC system is hyphenated to ICPMS because of band broadening effects resulting from the eluent introduction system into the ICP. The sample introduction system for the ICPMS, as described in the experimental section, consists of a simple cross flow nebulizer passing into the Peltier cooled spray chamber. This eluent introduction system is necessary when using chromatographic mobile phases because the organic component of the mobile phase has a cooling effect on the plasma. This cooling effect can compromise the plasma, making it work less efficiently or, in the worst case, stopping it working altogether. However, due to its volume (30 ml) the chamber undoubtedly acts as source of some peak broadening. To compensate the carrier gas pressure can be increased to move the vapour stream through more rapidly but this procedure also has an effect on the plasma so a balance has to be found between the chromatographic peak shape and the integrity of the plasma. The published LC–MS methods for the determination of CXB in plasma have LODs of 5 ng/ml [5] and 20 ng/ml [6]. In the case of EXB the published LC–MS methods report the determination of the drug in plasma samples over the range 5–2500 ng/ml (or 0.5–250 ng/mg for a ^{13}C-labelled version of the drug) [12], 0.2–200 ng/ml [13] and 10–2500 ng/ml [14], respectively. Clearly UPLC/ICPMS as reported here compares very well with these pre-existing methods and the current method provided more than adequate sensitivity and specificity for the determination of the two analytes in these samples, whilst also providing a wide linear working range.

Finally the ICP detector proved well able to cope with the UPLC system as the sampling rate was fast enough to collect enough data points across the peak even for the narrower peaks. Thus separations can be performed at low flow rate if high sensitivity is desired but also in higher rates if a short analysis time is desired employing the advantages of UPLC.

4. Conclusions

ICPMS coupled to UPLC provided a sensitive and selective method for the analysis of celecoxib and etoricoxib in the serum and synovial fluid of the patient population examined and has demonstrated for the first time the application of this approach for quantitative drug bioanalysis. The methodology for serum is comparable or better than that of the pre-existing methods, whilst the analysis of these analytes in synovial fluid has not previously been performed. The analysis of both serum and synovial fluid has demonstrated potential differences in the distribution of the two COX-2 inhibitors.

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