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Determination of lonazolac and its hydroxy and O-sulfated metabolites by on-line sample preparation liquid chromatography with fluorescence detection☆

Gerhard Friedrich[a,*], Thorsten Rose[a], Klaus Rissler[b]

[a]*Institute of Contract Research, Pharmbiodyn, P.O. Box 1108, D-79207 Denzlingen, Germany*
[b]*Ciba Specialty Chemicals Inc., K-402.3.34, CH-4002 Basel, Switzerland*

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Abstract

A reliable method, which can be used for the determination of lonazolac and its hydroxylated and O-sulfated metabolites in cell culture media with methyllonazolac as the internal standard is described. The procedure employs on-line sample enrichment using a BioTrap 500 MS™ (20×4 mm I.D.) extraction pre-column and subsequent gradient separation on an Xterra MS C_{18}-H™ (100×3 mm I.D., 3.5 μm particles) analytical column in the back-flush mode. Signal monitoring was done by measurement of fluorescence responses at 273 nm for excitation and 385 nm for emission. Structural identity of analyte peaks was confirmed by liquid chromatography coupled to mass spectroscopy (LC–MS–MS) using an electrospray ionization (ESI) source in the selected reaction monitoring (SRM) mode. Mean recoveries of lonazolac, hydroxylonazolac and lonazolac sulfate, respectively, from the biological matrix were 104.2±3.5, 96.7±2.2, and 100.9±3.5%. The limit of detection (LOD) for the three compounds was about 5 ng/ml using a total sample volume of only 50 μl. Linearity of signal responses versus concentration for all three analytes was accomplished in the range 10–600 ng/ml. The mean values of the coefficients of variation (C.V.) for quality control samples measured in duplicate at three different days at the 10, 40, 100, and 400 ng/ml level were 4.46±1.15, 3.94±2.13 and 4.79±2.07% for lonazolac, hydroxylonazolac and lonazolac sulfate. The target analytes were sufficiently stable at both storage and sample preparation conditions because no substantial deviations between analyte concentrations measured before and after subsequently performed freeze and thaw cycles were observed.

Keywords: Lonazolac; Hydroxylonazolac; Methyllonazolac

1. Introduction

As, for example, indomethacin and diclofenac, lonazolac [3-(*p*-chlorophenyl)-1-phenylpyrazole-4-acetic acid] (Fig. 1a) is an aryl-substituted acetic acid derivative and belongs to the family of non-steroidal anti-inflammatory drugs (NSAIDs). The pharmacological actions of these compounds is attributed to their inhibitory effects on the conversion of arachidonic acid into metabolites via the cyclo-oxygenase pathway. Lonazolac was found to possess high anti-inflammatory potency but less toxicity compared

☆This paper is dedicated to Professor Hinrich Cramer at the occasion of his 70th birthday.

*Corresponding author. Fax: +49-76-66-7255.
E-mail addresses: info@pharmbiodyn.com (G. Friedrich), klaus.rissler@cibasc.com (K. Rissler).

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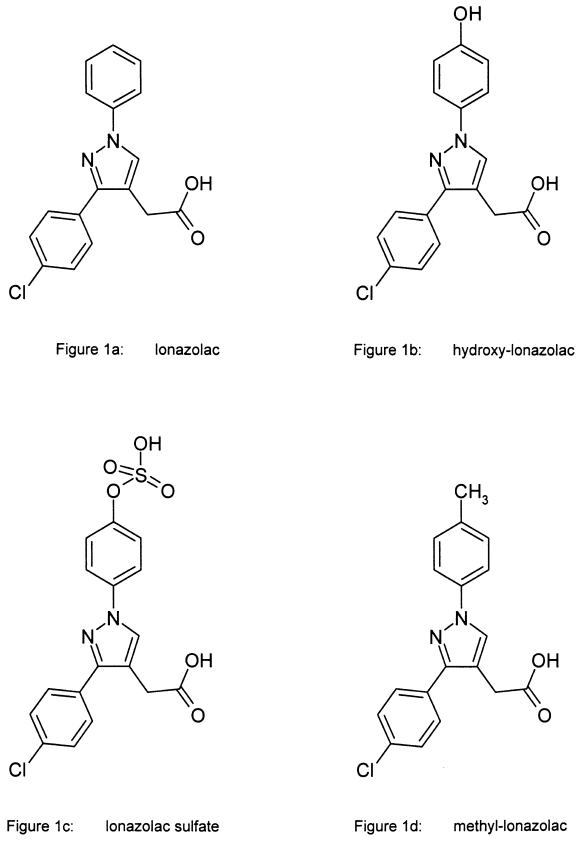

Fig. 1. (a) Structural formula of lonazolac, (b) hydroxylonazolac, (c) lonazolac sulfate, and (d) methyllonazolac, as the internal standard.

with other drugs [1] and undergoes degradation in the liver by cytochrome oxidase 2C9 (CYP 2C9) to the hydroxylated metabolite [3-(*p*-chlorophenyl)-1-(*p*-hydroxyphenyl)pyrazole-4-acetic acid] (Fig. 1b) which is further metabolized by kidney and liver arylsulfotransferase (AST) to the O-sulfated derivative [3-(*p*-chlorophenyl)-1-(*p*-hydroxyphenyl-sulfuric acid monoester)pyrazole-4-acetic acid] (Fig. 1c).

Although not abundant in the literature, several reports describe high-performance liquid chromatographic separation of lonazolac and its hydroxy metabolite. For the first time quantification of lonazolac and hydroxy-lonazolac in serum was reported by Huber et al. [2] in 1982 by HPLC applying a pre-column enrichment and column-switching procedure as also effected by Arnold et al. [3] for the analysis of lonazolac, pirenzepine, mefloquine and pentoxifylline in biological material. In both cases signal monitoring was done by measurement of fluorescence responses, whereas the latter authors additionally used UV detection at 282 nm. Further reports on determination of lonazolac in biological matrices by HPLC were published by Stehlik et al. [4] and Schulz and Schmoldt [5] by studying the plasma levels of some NSAIDs among them lonazolac with fluorescence [4] and UV [5] detection. Quantitative determination of lonazolac in urine samples with UV detection was published by Battista et al. [6]. Vinge and Bjoerkman [7] investigated the dependence of lonazolac and ibuprofen plasma concentrations on platelet aggregation applying fluorescence detection. A method for application of HPLC for rapid analysis of cytochrome P-450 mediated xenobiotic metabolism of lonazolac as well as the anti-parkinsonian drug bromlisurid and ethoxycoumarin, the latter compound being used as the classical substrate of enzymatic de-ethylation activity, was reported by Simon et al. [8] using direct injection of microsome suspensions and back-flush column-switching HPLC followed by UV detection. In more recent reports, strategies for efficient separation and detection of NSAIDs including lonazolac using stationary phases of different length and inner diameter were published by Baeyens et al. [9,10]. Furthermore, an application of HPLC for determination of the levels of lonazolac and its hydroxy metabolite in plasma, synovial fluid and synovial membranes was published by Deneke et al. [11]. Fluorescence detection was used by the latter authors and detection limits of 10 pg lonazolac were reported [9,11].

The primary goal for the quantitative determination of lonazolac, hydroxy-lonazolac and lonazolac sulfate was its application to the measurement of either cytochrome oxidase 2C9 (Cyp 2C9) or arylsulfotransferase (AST) activity in cell cultures, in particular with respect to the kinetics of the enzymatic reaction with lonazolac as a classical substrate for AST activity. For this purpose, a sensitive and reliable HPLC procedure with an integrated on-line sample pre-purification procedure preferably suited for processing of large sample numbers by means of column-switching and fluorescence detection was developed and methyl-lonazolac [3-(*p*-

chlorophenyl)-1-(*p*-methylphenyl)pyrazole-4-acetic acid] (Fig. 1d) used as the internal standard. Structural identity of the target compounds was furthermore confirmed by MS–MS detection in the selected reaction monitoring (SRM) mode using an ESI source ion-trap analyzer.

2. Experimental

2.1. Reagents and materials

Lonazolac, hydroxy-lonazolac and lonazolac sulfate as well as the internal standard methyl-lonazolac were kindly provided by Byk-Gulden Lomberg Pharmaceuticals (Konstanz, Germany). Methanol, 2-propanol, ammonium acetate, formic acid, ammonia (25% solution in water), all analytical grade, were obtained from Merck (Darmstadt, Germany). Triethylamine was purchased from Fluka (Buchs, Switzerland). The V79-hSULT1E1 cell line was a gift, kindly donated by Professor H.R. Glatt from the German Institute of Human Nutrition (DIfE), Department of Toxicology, (Potsdam-Rehbrücke, Germany). Dulbecco's modified Eagle medium (DMEM) was obtained from Cell Concepts (Umkirch, Germany). High purity water for the use in HPLC was prepared with a Milli-Q water system™ from Millipore–Waters (Milford, MA, USA). A BioTrap 500 MS™ pre-column (20×4 mm I.D.) from Chromtech (Hägersten, Sweden) and an Xterra MS-C_{18}-H™ (100×3 mm I.D, 3.5 μm particle size) analytical column from Waters (Milford, MA, USA) were used for sample extraction and separation, respectively.

2.2. Preparation of calibration samples, internal standard and quality controls

Stock solutions of 10 μg/ml lonazolac, hydroxy-lonazolac and lonazolac sulfate as well as of methyl-lonazolac as the internal standard were prepared in methanol–water (1:1, v/v) and stored at −20°C. A working solution containing 400 ng/ml of methyl-lonazolac was daily prepared by removal of 400 μl from the corresponding stock solution and dilution to a final volume of 10 ml with 1 *M* triethylammonium formate (TEAF) prepared from triethylamine adjusted to a pH value of 5.5 with concentrated formic acid. A working solution (2 μg/ml) of lonazolac, hydroxy-lonazolac and lonazolac sulfate was daily prepared by five-fold dilution of the stock solution (10 μg/ml) with a medium containing four volume parts of 1 *M* TEAF (pH 5.5) and one volume part of DMEM. The following final calibration range was obtained by serial dilution of this standard working solution with the same medium yielding 10, 20, 30, 50, 100, 200, 300, and 600 ng/ml to which internal standard solution yielding a final concentration of 200 ng/ml was added. For determination of the % recovery, the ratio of the peak areas measured in the biological matrix to those in buffer only at three different concentrations, i.e. 80, 100 and 120 ng/ml using the column-switching technique described below, was chosen. Quality control samples (QCs) were prepared from separately prepared stock solutions similarly to the calibration standards yielding final concentrations of 10, 40, 100 and 400 ng/ml, measured in duplicate at three different days. In accordance with the calibrator solutions, all QCs were spiked with internal standard also yielding a final concentration of 200 ng/ml. Freeze and thaw stability investigations were done at concentrations of 10 ng/ml for the lower limit of quantification (LLOQ) and 600 ng/ml for the higher limit of quantification (HLOQ) in duplicate at three different days.

2.3. Sample extraction and chromatographic separation

An on-line pre-column enrichment column-switching HPLC procedure operated in the back-flush mode was used for sample preparation. In this respect, an artificial sample containing lonazolac, hydroxy-lonazolac, lonazolac sulfate and methyl-lonazolac as the internal standard dissolved in 1 *M* TEAF (pH 5.5)–DMEM (4:1, v/v) was first centrifuged for 5 min at 2000 *g* and the supernatant extracted by means of Biotrap 500 MS™ precolumn cartridges. In addition, an incubation mixture consisting of V79-h-SULT1E1 cells, to which hydroxy-lonazolac as the substrate for AST activity measurements had been added was treated correspondingly. Sample application, pre-purification and concentration as well as final elution onto the analytical column by the used

column switching technique is depicted in Fig. 2. In order to provide a better insight into the whole procedure, the time-dependent analytical events for the two pairs pre-column 1/analytical column 1 (PC-1/AC-1) and pre-column 2/analytical column 2 (PC-2/AC-2) were additionally illustrated by means of Table 1. The switching technique comprises two Vici six-port and two Vici ten-port valves obtained from Valco Europe (Schenkon, Switzerland) for the sample extraction — back-flush process involving PC-1 and PC-2 for sample extraction and pre-concentration and AC-1 and AC-2 for the analytical separation procedure including cleaning and re-equilibration of either pre-columns or analytical columns as well as cleaning of the ion-source. Briefly, a 50-μl aliquot was aspirated into the sample loop by use of a type AS 3000 autosampler from Thermo Separation Products (San José, CA, USA) and injected onto PC-1 with a type LKB 2150 pump (pump C) from Pharmacia-LKB (Bromma, Sweden) with 10 m*M* TEAF (pH 5.5) prepared from triethylamine adjusted to the final pH of 5.5 by addition of concentrated formic acid (extraction buffer). Upon flushing with this buffer at a flow-rate of 3.2 ml/min, the compounds of interest were quantitatively retained on the extraction pre-column, whereas the large amount of potential interfering components arising from impurities of the medium, such as, e.g. proteins and others, were eluted to waste. After an extraction interval of 1 min both the

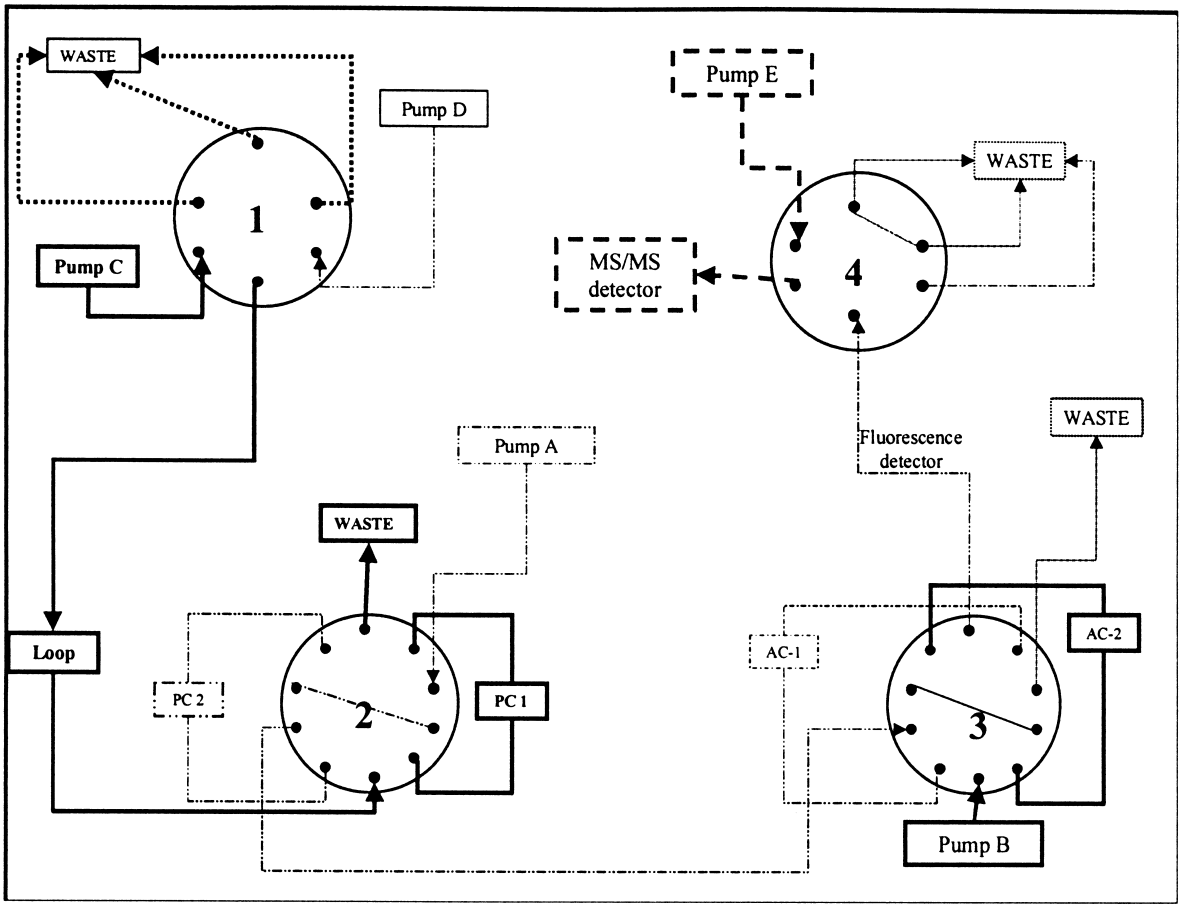

Fig. 2. Schematic representation of the on-line sample preparation column-switching system for sample enrichment and chromatographic separation.

Table 1
Different time-dependent analytical events used in the column-switching procedure

Time (min)	Analytical events on PC-1/AC-1	Analytical events on PC-2/AC-2	MS–MS events
0–1	Sample injection–sample extraction on PC-1 with m*M* TEAF (pH 5.5) by means of pump C at 3.2 ml/min	Start cleaning of AC-2 with methanol–water 1:1 (v/v) by means of pump B at 0.2 ml/min	Cleaning ion-source with methanol–water 1:1 (v/v) at a flow-rate of 0.2 ml/min by means of pump E.
1–6	Back-flush PC-1/AC-1 and begin of analytical separation with pump A at 0.2 ml/min (conditions)	Cleaning loop and PC-2 by means of pump D with methanol–water 1:1 (v/v) at 0.2 ml/min Cleaning of AC-2 with methanol–water 1:1 (v/v) by means of pump B continued	Cleaning ion-source continued
6–12	Analytical separation on AC-1 continued	Cleaning loop and PC-2 continued End of cleaning AC-2 (10 min) and start equilibration of AC-2 with 70% 10 n*M* ammonium acetate (pH 8.5) and 30% methanol–2-propanol (90:10, v/v) at 0.2 ml/min by means of pump B	Segment m/z 408→m/z 328 Segment m/z 328→m/z 284
12–18	Analytical separation on AC-1 continued	Cleaning loop and PC-2 continued Equilibrium of AC-2 continued	Segment m/z 312→m/z 268 Segment m/z 326→m/z 282
18–19	Analytical separation on AC-1 continued	Equilibration of PC-2 with 10 m*M* TEAF (pH 5.5) by means of pump C at 3.2 ml/min Equilibration of AC-2 continued	
19–20	End analytical separation	End equilibration of PC-2 End equilibration of AC-2	Cleaning of ion source by means of pump E

Pump A=elution pump; pump B=cleaning and equilibrating pump; pump C=extraction pump; pump D=cleaning pump; pump E=ion source cleaning pump.

six-port valve 1 and the ten-port valve 2 were simultaneously switched. This measure effects substances retained on PC-1 to be eluted in the opposite direction, i.e. ''back-flushed'' onto AC-1 for subsequent separation by means of pump A. Due to the fact that the compounds of interest do not leave the column within the first 6 min, the whole column efflux is diverted to waste during this time interval, while in parallel, the ion source is cleaned with methanol–water (1:1, v/v) at a flow-rate of 0.2 ml/min. by means of a type SP 8810 isocratic pump (pump E) from Spectra Physics (San José, CA, USA). During the analytical run on AC-1 cleaning of both PC-2 and AC-2 is separately effected with methanol–water (1:1, v/v) using a type LKB 2150 pump (pump D) from Pharmacia-LKB for PC-2 and a type SP 8800 ternary gradient pump (pump B) from Spectra Physics for AC-2 followed by equilibration with either extraction buffer (PC-2) using pump C and eluent at the starting conditions of analytical separation (AC-2) using pump B (see Fig. 2 and Table 1). Analytical separation was performed with a type P 2000 binary gradient pump from Thermo Separation Products (San José, CA, USA) (pump A) on an Xterra MS C_{18}-H™ column and gradient elution (gradient program, see Table 2) with 10 m*M* ammonium acetate adjusted to pH 8.5 with ammonia (25% in water) as mobile phase A and methanol–2-propanol (9:1, v/v) as mobile phase B at a flow-rate of 0.2 ml/min. Signal monitoring was done by measurement of the fluorescence responses with a type F 1100 fluorescence detector from Hitachi (Tokyo, Japan) at 273 nm for excitation and

Table 2
Gradient program for separation on the analytical column

Time (min)	Mobile phase A	Mobile phase B
0	70	30
2	70	30
2.5	40	60
13	40	60
18	0	100
20	0	100

385 nm for emission. Besides determination of analytes by means of fluorescence detection, LC–MS–MS in the selected reaction monitoring (SRM) mode was applied for structural confirmation.

2.4. LC–MS conditions

The LC–MS–MS analysis was carried out on a type LCQ™ ion-trap mass spectrometer purchased from ThermoQuest Finnigan (Bremen, Germany) equipped with an electrospray ionization (ESI) source operated in the selected reaction monitoring (SRM) mode. Mass spectra were obtained in the negative ion mode applying a source voltage of 5.0 kV. The heated capillary temperature was set up to 240°C, while the settings for sheath and auxiliary gas (in both cases nitrogen, purity degree 99.999%) were adjusted to 60 and 20 units, respectively. Data acquisition was done by monitoring the loss of a carbon dioxide molecule from the carboxymethyl substituent at 4-position of the central pyrazole ring system, i.e. m/z 312→268 for lonazolac, m/z 328→284 for hydroxy-lonazolac, m/z 408→364 for lonazolac sulfate and m/z 326→282 for methyl-lonazolac as the internal standard.

3. Results

3.1. Pre-column sample extraction, chromatographic separation and detection

As revealed in Figs. 3 and 4 showing the traces from the fluorescence responses of a mixture of lonazolac, hydroxy-lonazolac, lonazolac sulfate and internal standard in medium (Fig. 3) and a biological sample, i.e. medium containing the V79-hSULT1E1 cell line, to which hydroxy-lonazolac as the substrate for arylsulfotransferase activity measurements[1] and internal standard were externally added and incubated for 24 h (Fig. 4), baseline separation of all compounds of interest was achieved using on-line sample extraction by means of the BioTrap 500

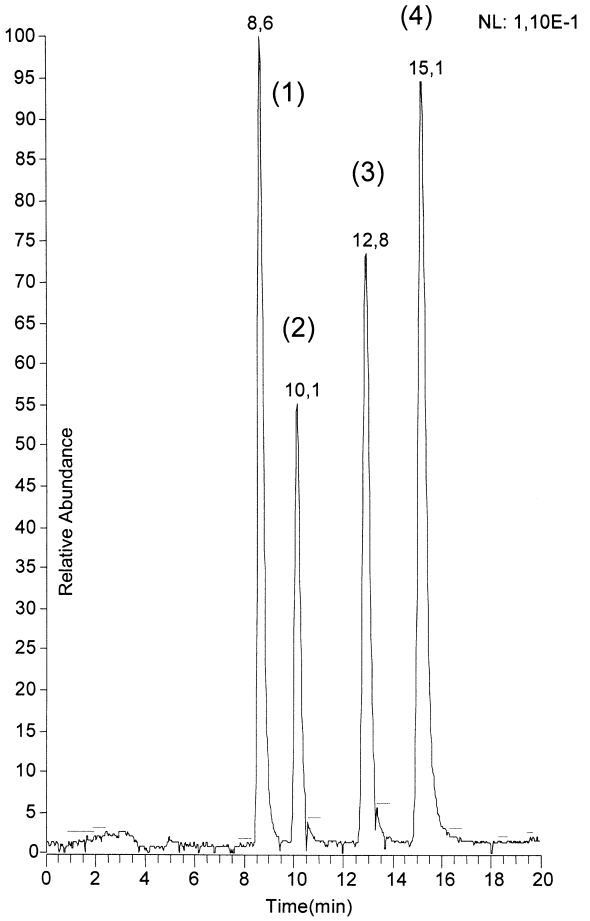

Fig. 3. Chromatogram (fluorescence detection) of a mixture consisting of each 100 ng/ml lonazolac sulfate (1), hydroxy-lonazolac (2), lonazolac (3) and methyl-lonazolac (4) as the internal standard in a medium containing four volume parts of 1 *M* TEAF (pH 5.5) and one volume part of DMEM (for details, see Experimental).

MS™ pre-column and subsequent back-flush onto the analytical Xterra MS C_{18}-H™ column (scheme depicted in Fig. 2). It is conspicuous that no measurable peak broadening occurred compared with direct injection of the same analyte mixture without pre-concentration step (results not shown) and thus underlines the feasibility of the new experimental design involving the back-flush column-switching technique. The limits of detection and quantification of about 5 ng/ml for the three compounds obtained

[1]The details of arylsulfotransferase determination exceed the scope of this report and thus will be published in a separate paper.

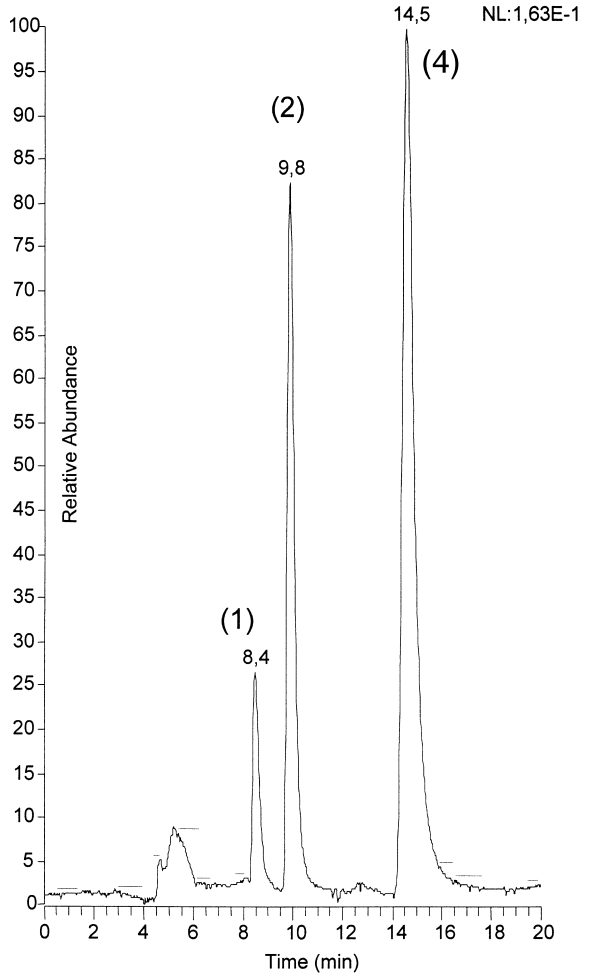

Fig. 4. Chromatogram (fluorescence detection) of a biological sample, i.e. medium containing 10^6 V79-hSULT1E1 cells to which hydroxy-lonazolac (10 μM) as the substrate for arylsulfotransferase (AST) activity measurements (t=24 h) was added (peak assignment as in Fig. 3).

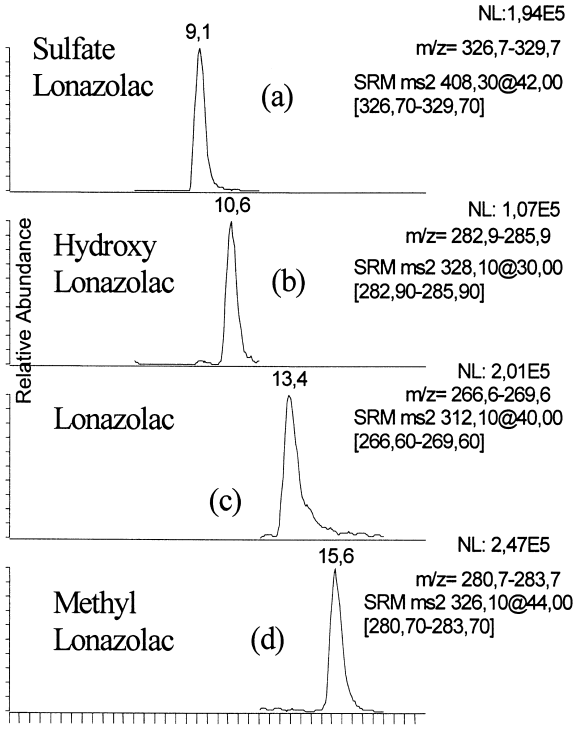

Fig. 5. Reconstructed ion chromatograms obtained from mass spectroscopy in the SRM mode (RIC trace) of the separately injected lonazolac-sulfate (a), hydroxy-lonazolac (b), lonazolac (c) and methyl-lonazolac (d) as the internal standard all dissolved in a medium containing four volume parts of 1 *M* TEAF (pH 5.5) and one volume part of DMEM (for details, see Experimental).

with this method are sufficient for most applications and thus fulfill the requirements of determination of the target analytes in culture media.

3.2. LC–MS investigations

As revealed in Fig. 5a–d showing the reconstructed ion chromatograms (RIC) obtained from mass spectroscopy in the SRM mode of the separately injected target analytes, unequivocal structural confirmation of lonazolac, hydroxy-lonazolac, lonazolac sulfate and methyl-lonazolac is achieved. However, it is surprising that sensitivity of mass spectroscopy is slightly lower than that observed with fluorescence detection, but nevertheless proved to be sufficient in order to cover the expected concentration range of these compounds for their determination in biological matrices.

3.3. Recovery

Recovery values, which have been determined using the peak area ratios obtained from the three target compounds as well as from the internal

Table 3
Precision and accuracy data of (a) lonazolac, (b) hydroxy-lonazolac, and (c) lonazolac sulfate at four different concentrations measured in duplicate at three different days

	Nominal concentration (ng/ml)			
	10	40	100	400
(a) Lonazolac				
Mean	11.47	41.33	103.81	417.12
SD	0.57	2.49	3.38	15.92
C.V. (%)	4.97	5.81	3.25	3.82
Accuracy (%)	114.67	103.32	103.81	104.28
(b) Hydroxy-lonazolac				
Mean	10.53	39.98	99.84	404.61
SD	0.62	2.24	2.64	6.59
C.V. (%)	5.89	5.60	2.65	1.63
Accuracy (%)	105.27	99.94	99.84	101.15
(c) Lonazolac sulfate				
Mean	10.84	40.51	102.49	406.50
SD	0.84	1.78	3.03	16.39
C.V. (%)	7.75	4.40	2.96	4.03
Accuracy (%)	108.38	101.27	102.49	101.63

standard in the biological matrix to those of them dissolved in buffer only at three different concentrations, i.e. 80, 100 and 120 ng/ml, measured in quintuplicate, were 104.2±3.5, 96.7±2 2, 100.9±3.5 and 105.2±2.6%, respectively, for lonazolac, hydroxy-lonazolac, lonazolac sulfate and the internal standard methyl-lonazolac.

3.4. Linearity

Linearity for lonazolac, hydroxy-lonazolac and lonazolac sulfate, respectively, was achieved in the concentration range of 10–600 ng/ml including eight external standards (for individual concentrations, see Experimental).

3.5. Specificity

As shown in Fig. 4, complete absence of matrix effects was verified and thus interfering compounds from the used medium can extensively be ruled out.

3.6. Precision and accuracy

In preliminary experiments in order to provide an initial test of the feasibility of the method for determination of lonazolac, hydroxy-lonazolac and lonazolac sulfate for measurement of AST and Cyp 2C9 activity, precision and accuracy have been determined using internal standardization whereby linear regression was used to fit lines to the data. The mean values for the correlation factors R^2 for lonazolac, hydroxy-lonazolac and lonazolac sulfate were 0.9948, 0.9984 and 0.9979, respectively. Table 3a–c shows the data obtained from the three analytes measured at four different concentrations in duplicate at three different days. The values for the coefficient of variation as well as accuracy of QCs measured at the 10, 40, 100 and 400 ng/ml level ranged from 0.6 to 5.8% and 103.3 to 114.7%, 1.6 to 5.9% and 99.8 to 105.3%, as well as 3.0–7.8% and 101.3–108.4%, respectively, for lonazolac, hydroxy-lonazolac and lonazolac sulfate.

3.7. Freeze and thaw stability

As can be seen from Table 4, no marked detrimen-

Table 4
Freeze and thaw stability of lonazolac, hydroxy-lonazolac and lonazolac sulfate at two different concentrations in duplicate measured at three different days

	Nominal concentration (ng/ml)					
	Lonazolac		Hydroxy-lonazolac		Lonazolac sulfate	
	10	600	10	600	10	600
Mean	9.92	601.52	10.31	595.92	10.35	602.13
SD	0.49	17.60	0.58	15.26	0.29	18.91
C.V. (%)	4.95	2.93	5.63	2.56	2.82	3.14
Accuracy (%)	99.21	100.25	103.11	99.32	103.47	100.36

tal effect of three freeze–thaw cycles (thawing from −20°C to room temperature) performed at 10 ng/ml (LLOQ) and 600 ng/ml (HLOQ) in duplicate over an interval of 3 days was recognizable and thus underlines the stability of the drug under real-life conditions.

4. Discussion

4.1. Chromatographic separation, pre-column sample extraction and detection

In the hitherto published work for analysis of lonazolac and its hydroxy metabolite primarily reversed-phase HPLC using hydrophobic stationary phases, i.e. C_8 and C_{18} materials and gradient elution with phosphate-buffered aqueous acetonitrile systems were applied [3–5,9,10], whereas other authors used sulfuric acid [2] and sodium acetate buffers [6] for pH adjustment of the mobile phase. However, due to their content of low volatile buffer additives, these mobile phases are not compatible with subsequent on-line mass spectroscopic measurements (LC–MS hyphenation) and therefore require the use of more volatile eluent systems. In preliminary attempts of mobile phase optimization in order to achieve both satisfactory separation of the target analytes and potential interfering components arising from the biological matrices, a slightly acidic eluent was successfully applied. Unfortunately, these conditions exerted a detrimental influence in combination with LC–MS–MS resulting in marked signal depression, which however could be efficiently counterbalanced by a switch to a slightly alkaline mobile phase for analytical separation (pH 8.5) with a stationary phase being stable under the conditions of long-term application. For this reason, in addition to the determination of the target analytes by measurement of their fluorescence responses, mass spectroscopy performed in the highly specific selected reaction monitoring (SRM) mode may also be applied.

From a practical point of view in order to simplify and speed up sample preparation and thus to achieve high sample throughput within a short time period, it was decided to apply the pre-column enrichment procedure. This technique taking advantage of the stationary phase chemistry usually applied in RP-HPLC but using more course materials in the 25–40-μm particle size range was first published by Roth et al. in 1981 [12] and proved to be a rapid, efficient, reliable and reproducible alternative to the more conventional liquid–liquid extraction, protein-precipitation, protein-precipitation liquid–liquid extraction and even ''off-line'' solid-phase extraction (SPE) procedures. From a variety of different adsorbents tested for pre-column sample extraction the BioTrap 500 MS™ material proved to have optimum properties for either efficient sample trapping or recovery. It belongs to the family of restricted access media (RAM), which are characterized by a bio-compatible hydrophilic outer and a hydrophobic inner (pore) surface. As a consequence, it permits large-sized polar analytes, such as, e.g. proteins, glycoproteins, polysaccharides and, in addition, also inorganic sample constituents embedded within biological matrices to be rapidly swept out while completely retaining less polar small-sized xenobiotics by hydrophobic interactions with the non-polar inner core of the adsorbent. Nevertheless, due to its high polarity, lonazolac sulfate was only weakly retained when dissolved in the generally used buffer solutions, whereas in contrast, lonazolac and its hydroxylated metabolite were completely retained under these conditions. The problem of poor retention and thus substantial breakthrough of lonazolac sulfate was successfully overcome by sample application in a small-sized and thus sufficiently volatile ion-pairing reagent, i.e. 1 *M* triethylammonium formate (pH 5.5) and removal of possibly interfering matrix components by flushing the extraction pre-column with 10 m*M* TEAF (pH 5.5). In fact, due to ion-pairing with an organic counter-ion, a substantial gain in overall hydrophobicity of lonazolac sulfate was achieved, resulting in quantitative retention on the BioTrap 500 MS material. The relatively high flow-rate of 3.2 ml/min applied for pre-column flushing was chosen according to the supplier's application manual recommending this flow-rate in the case of short flush times. It should be emphasized however, that analytical separation is often associated with relatively broad and sometimes distorted peaks when inappropriate mobile phases were used for the transfer from the extraction pre-column (PC) to the analytical column (AC) during the course of the back-flush procedure. In order to circumvent this

undesired phenomenon and, in addition, to produce sharp peaks and thus lower detection limits, back-flush of the analyte from PC to AC was done with a mobile phase yielding rapid desorption of trapped sample from the PC at conditions, which are still insufficient for its elution from the AC. This aim was achieved with an eluent rich in aqueous buffer, consisting of mobile phase A (10 m*M* ammonium acetate, pH 8.5) and mobile phase B (methanol–2-propanol, 90:10, v/v) at a volume ratio of 70:30 for 2 min effecting complete analyte transfer from PC to AC. Final separation was accomplished by a switch to 40% mobile phase A and 60% mobile phase B (v/v) within the next 0.5 min (i.e. 2–2.5 min), isocratic elution at these conditions for another 10.5 min (i.e. from 2.5 to 13 min), subsequent gradient elution to a final content of 100% mobile phase B (13–18 min) and a hold at these eluent composition for another 2 min (see gradient program in Table 1). As a consequence of this sophisticated on-line sample extraction and pre-purification design, the analyte molecules to be determined are focused as a small band at the head of AC, which permits their elution as sharp and symmetrical peaks resulting in sensitive detection.

Although the lonazolac structure possesses two aromatic ring systems and thus spectrophotometric detection would be the first choice, determination of concentrations in the lower ng/ml range cannot be realized. However, either parent compound or the metabolite hydroxy-lonazolac and its conjugate lonazolac sulfate as well as the internal standard exhibit satisfactory fluorescence properties in order to achieve the goal of reliable detection in the lower ng/ml range as often encountered in biological matrices. Baeyens et al. [10] as well as Deneke et al. [11] reported limits of detection (LOD) of 10 pg/ml when using fluorescence detection. However, considering a detection limit of 100 ng/ml as found by Simon and Hopley [8] with measurement of UV absorption at 280 nm, the corresponding values of 10 pg/ml seem to be at least one order of magnitude too low because those LODs are only realizable with laser induced fluorescence (LIF) and electrochemical detection the latter typically encountered in the analysis of catecholamines. In this respect, detection limits for lonazolac and hydroxy-lonazolac of 7 and 3 ng/ml, respectively, as reported by Vinge and Bjorkman [7] also using fluorescence detection, appear to be more realistic and thus are in good agreement with our findings of 5 ng/ml using a 50-μl sample. Nevertheless, it may be stated that the LODs found in the present investigations should be further lowered by injection of higher sample volumes.

At first sight, the column-switching procedure occurs to be rather complicated and thus may preclude its use in a number of laboratories because of the lack of appropriate instrumentation. However, it should be emphasized that a lot of problems usually associated with column-switching techniques, such as, e.g. carry-over effects producing unsatisfactory reproducibility is completely eliminated due to extensive cleaning of either sample pre-purification/analytical separation system or ion-source prior to injection of another sample. Once established, the method permits the non-perturbed, reliable and reproducible ‘‘on-line’’ extraction and final separation of large sample numbers and thus overcomes the drawbacks of the more conventionally and time-consuming alternatives, such as, e.g. liquid–liquid extraction and solid-phase extraction (SPE), which are only hardly applicable in the on-line mode. With an overall analysis time of 20 min it is possible to investigate about 50 samples overnight and in our laboratory practice up to more than 60 samples have been measured in one chromatographic run, which underlines the substantial advantages of this technique.

4.2. Recovery, linearity, precision and accuracy

As can be concluded from the data of preliminary experiments compiled in Tables 3a–c and 4, the applied experimental design should be applicable for determination of concentrations of lonazolac, hydroxy-lonazolac and lonazolac sulfate in biological media, as, e.g. encountered in measurements of AST and Cyp 2C9 activity. In addition, repeatedly performed freeze and thaw cycles did not prove to have substantial impact on the measured analyte concentrations, which furthermore underlines the feasibility of the procedure under conditions of practical use.

5. Conclusions

A sensitive on-line sample enrichment procedure based on column-switching high-performance liquid chromatography for the use of determination of lonazolac, hydroxylonazolac and lonazolac sulfate in cell culture media is reported. The technique takes advantage of sample concentration on a bio-compatible pre-column allowing interfering compounds to be swept out while quantitatively retaining the components of interest. After back-flushing the trapped analyte molecules onto an analytical column, which is stable up to pH values of about 12, separation is effected by means of a slightly alkaline (pH 8.5) mobile phase and measurement of signal responses by fluorescence detection at 273 nm for excitation and 385 nm for emission yielding a detection limit of about 5 ng/ml. As a consequence, the extensively automated procedure allows processing of large sample numbers making any time-consuming extraction and back-extraction steps unnecessary. The choice of an alkaline mobile phase is recommendable because marked signal depression in LC–MS–MS experiments occurs when applying the preponderantly used acidic mobile phases. Although seeming rather complicated at first sight, the assay permits rapid processing and quantification of large sample numbers and thus may be regarded as an economical alternative to the more conventional time-consuming procedures. In conclusion the new experimental design should also be applicable for determination of levels of a wide variety of biologically active compounds including either parent drug and/or metabolites in plasma, urine and tissue after appropriate changes in conditions of either pre-column sample enrichment or subsequent chromatographic separation.

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