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Analytica Chimica Acta 492 (2003) 253–260
ANALYTICA CHIMICA ACTA
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Determination of ropivacaine and its metabolites in patient urine: Advantage of liquid chromatography–tandem mass spectrometry over liquid chromatography–UV detection and liquid chromatography–mass spectrometry

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Accepted 13 May 2003

Abstract

Analysis of urine samples from a clinical study of ropivacaine and its metabolites, 3-hydroxyropivacaine (3-OH-ropivacaine) and PPX, by an LC–UV method showed high concentrations of 3-hydroxyropivacaine, 2–50 times higher than expected. In the study, the patients were treated with a number of drugs in combination with ropivacaine. These drugs were paracetamol, lidocaine, fentanyl, morphine and trimethoprim. When the fraction of 3-hydroxyropivacaine was collected from LC–UV and analysed by LC–MS, only a high signal at mass number 291 [3-hydroxyropivacaine (MH^+)] was observed. This observation indicates that it may be a drug or a metabolite having the same mass number as 3-hydroxyropivacaine and eluting at the same retention time on the LC system that gives a high signal in UV and MS detection. The examination of the drugs given showed that trimethoprim has the same molecular weight as 3-hydroxyropivacaine. The analysis of trimethoprim by LC–UV and LC–MS showed that under the given conditions it has the same retention time as 3-hydroxyropivacaine. The tuning of 3-hydroxyropivacaine and trimethoprim by MS–MS showed that both substances have the same precursor ions (*m*/*z*: 291) but different product ions (*m*/*z*: 126 and 123 for 3-hydroxyropivacaine and trimethoprim, respectively). This study shows that the use of LC–MS–MS may lead to more reliable results than LC–UV and LC–MS.

Keywords: Urine samples; Ropivacaine, 3-OH-ropivacaine; Trimethoprim; LC–UV; LC–MS–MS

1. Introduction

Mass spectrometry (MS) is at present one of the most powerful analytical techniques available, particularly in pharmaceutical analysis, where good selectivity and high sensitivity are often needed. The more recent developments in ionisation technologies make mass spectrometry an important tool for biological research. In the pharmaceutical industry measurements of drugs and metabolites in plasma are most important for drug discovery and development. The more accurate and rapid these measurements, the more quickly drugs make progress towards regulatory approval. Improvements in the technique of LC–MS during the past few years has led to decreased analysis times and increased throughput in the bioanalytical field. The use of LC–MS has strongly increased for

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doi:10.1016/S0003-2670(03)00574-9

applications involving biological samples [1–10]. The advent of modern, user-friendly mass spectrometers has led to a reconsideration of the application of mass spectrometry in the analytical process. In many instances this re-evaluation has resulted in an explosive increase in the use of the technique in industry, particularly for drug discovery—pharmacological and genomic—proteomic applications.

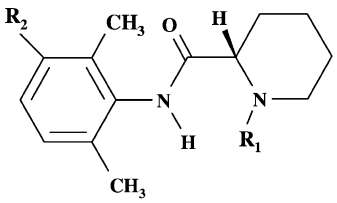
The new mass spectrometry technologies such as matrix-assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI) have simplified the analysis of proteins, peptides and drug metabolites. In general, low detection limits ranging from the picomole to the femtomole level are achieved.

The present study demonstrates the necessity of using the selective LC–MS–MS technique for biological samples from patients who are treated with several drugs.

Ropivacaine is a relatively new amide-type local anaesthetic, mainly used for surgery and for postoperative pain relief. In addition, it has a lower central nervous and cardiotoxic potential than its predecessor, bupivacaine [11]. The major metabolites of ropivacaine are PPX and 3-hydroxyropivacaine (3-OH-ropivacaine). The determination of ropivacaine and its metabolites has been performed by liquid and gas chromatography [12–14].

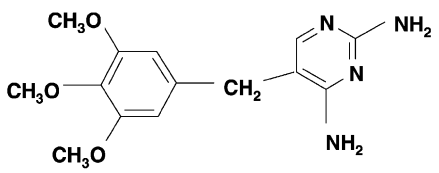
The aim of this study is to resolve a particular problem in one of our clinical studies for the analysis of ropivacaine and its metabolites in urine samples. The problem is that the concentration of one of metabolite (3-OH-ropivacaine) was 2–50 times higher than expected. Our hypothesis was that one or more of administrated co-drugs or their metabolites might interfere with 3-OH-ropivacaine giving high response in UV detector. To investigate this hypothesis the fraction of the 3-OH-ropivacaine peak was screened by mass spectrometry.

(A)



Compound	R_1	R_2
Ropivacaine	C_3H_7	H
3-OH-ropivacaine	C_3H_7	OH
PPX	H	H
Isopropyl-PPX(IS)	$CH(CH_3)_2$	H
$[^2H_7]$-ropivacaine	$C_3{}^2H_7$	H

(B)



Trimethoprim

Fig. 1. The structure of ropivacaine and its metabolites (A) and trimethoprim (B).

2. Experimental

2.1. Chemicals

Ropivacaine, metabolites and internal standard were supplied by the Department of Medicinal Chemistry, AstraZeneca (Södertälje, Sweden), as hydrochlorides. Acetonitrile (gradient grade), methanol LiChrosolv and formic acid (puriss >99%) were obtained from Merck (Darmstadt, Germany). Isopropyl-PPX and $[^2H_7]$-ropivacaine were used as internal standards for LC–UV and LC–MS/LC–MS–MS, respectively. The structures of ropivacaine, PPX, 3-OH-ropivacaine and internal standards are shown in Fig. 1A.

2.2. Apparatus

The LC–UV system consisted of two Shimadzu LC10ADvp pumps (Shimadzu corporation, Kyoto, Japan), an autosampler, CMA200, obtained from CMA/Micro-dialysis (Stockholm, Sweden) and UV detector (Spectra 100 UV-Vis, Spectra Physics,

San José, CA, USA) operated at 220 nm. Gradient HPLC was used with a mixer volume of 0.5 ml. Mobile phase A was 10 mM octanesulphonic acid in acetonitrile + phosphate buffer pH 2, $I = 0.05$ (3 + 97 (v/v)), and mobile phase B consisted of 10 mM octanesulphonic acid in acetonitrile + phosphate buffer pH 2, $I = 0.05$ (50 + 50 (v/v)). The gradient started from 30% of phase B for 10 min and then went up to 54% from 10 to 40.1 min. It increased directly to 90% in the space of 0.1 min and from 40.2 to 43.1 min was isocratic at 90% of phase B, and at 43.1 min phase B was set to 30% again. The flow rate was 1.0 ml/min. For the sake of system stability, 25 min was allowed for equilibration prior to the next injection. The analytical column was a Symmetry C8 (150 mm × 3.9 mm, 5 μm) and was purchased from Waters (Milford, MA, USA) the column temperature being 45 °C. An Optiguard (C8, 1 mm × 10 mm) obtained from Optimise Technologies (OR, USA) was used as a guard column.

The LC–MS system: the HPLC apparatus consisted of two pumps, Shimadzu LC10ADvp, Shimadzu corporation (Kyoto, Japan) and an autosampler, CTC-Pal, obtained from CTC Analytics AG (Zwingen, Switzerland). All experiments were conducted using a triple quadrupole mass spectrometric instrument (Micromass, Manchester, UK) equipped with a Z-electrospray interface (ESI) operating in positive ion mode. The source block and desolvation temperatures were 150 and 250 °C, respectively. Nitrogen was used as both drying and nebulising gas, while argon was used as collision gas. The capillary voltage was 3.1 kV and the sampling cone voltage was 38–40 V. The eluate from the analytical column was introduced into the ESI source after 3 min using the Valco valve. The data were collected using MassLynx version 3.1. All calculations were based on peak-area ratios. Prior to each batch of analysis, a test sample containing all the metabolites was analysed in order to check the sensitivity and to set integration parameters. The scan mode was multiple reaction monitoring using the precursor ion at m/z ($M + 1$) (m/z: 275, 233, 291) and after collisional dissociation the product ions m/z: 126, 84 and 126 were used for the quantification of ropivacaine, PPX and 3-OH-ropivacaine, respectively.

Gradient HPLC was used with a mixer volume of 0.5 ml. Mobile phase A was 10 mM ammoniumformate buffer (pH 3.9) in acetonitrile and water (10 + 90 (v/v)) and mobile phase B consisted of 10 mM ammoniumformate buffer (pH 3.9) in acetonitrile and water (80 + 20 (v/v)). The gradient started from 10% of phase B for 4 min and then went up to 50% from 4 to 15 min. It increased directly to 80% in the next 0.1 min and from 15.1 to 18 min was isocratic at 80% of phase B, and at 18.1 min phase B was set to 10% again. The flow rate was 0.35 ml/min. For the sake of system stability, the next injection was performed after 25 min. The analytical column was a YMC basic, 150 mm × 3.0 mm, 3 μm, and was purchased from YMC Europe GMBH (Schermbeck, Germany). The column temperature was 23 °C. An Optiguard (C8, 1 mm × 10 mm) obtained from Optimise Technologies Inc. (OR, USA) was used as a guard column. A VICI Valco C4W valve (VICI Valco Instrument Inc., Houston, USA) was used between the analytical column and the mass spectrometer.

2.3. Sample preparation

2.3.1. Hydrolysis

Due to the presence of conjugated metabolites, the total concentration of ropivacaine and its metabolites was determined after hydrolysis of urine samples with 6 M HCl (1.0 + 1.0 ml), 0.1 ml of internal standard was added and the mixture was then placed in a water bath at 95 °C for 1 h.

2.3.2. Solid-phase extraction (SPE)

A solid-phase extractor (ASPEC) was used. Hydrolysed urine sample of 0.250 ml was diluted with 5.0 ml (for LC–MS) or 1.0 ml (LC–UV) of water and 1.10 ml of the diluted sample was placed on a solid-phase column (Bond Elut SCX, 100 mg). Before application of the sample, the column was conditioned first with 2.0 ml methanol followed by 1.0 ml phosphate buffer (pH 2) and after application of the sample, the column was washed with 4.0 ml (methanol + phosphate buffer pH 2, 1 + 1). The analytes were eluted with 2.0 ml of ammonia + methanol (1 + 4). The eluate was evaporated and redissolved in 200 μl of mobile phase A.

2.4. Validation

Calibration standard solutions in urine were prepared. A standard curve with one zero concentration and at least seven standard concentrations was

Table 1
The accuracy and precision at various concentrations for LC–UV method

Compounds	Concentration (μM)	Accuracy (%, $n = 18$)	Precision R.S.D. (%) inter-assay ($n = 18$–24)
Ropivacaine	19.0	101	0.5
	3.9	100	0.8
	1.9	100	1.4
3-OH-ropivacaine	103	100	1.0
	8.2	99	1.1
	4.1	96	2.9
PPX	43.7	100	0.9
	7.3	102	1.3
	3.6	102	2.9

Table 2
The accuracy and precision at various concentrations for LC–MS–MS method

Compounds	Concentration (μM)	Accuracy (%, $n = 18$)	Precision R.S.D. (%)	
			Intra-assay ($n = 6$)	Inter-assay ($n = 18$)
Ropivacaine	0.56	98	4.9	5.9
	4.46	99	1.8	2.1
	16.7	98	2.6	2.6
3-OH-ropivacaine	3.38	99	4.2	5.9
	25.4	98	2.6	2.2
	102	98	3.2	3.2
PPX	1.52	96	7.1	7.3
	12.2	99	2.4	2.6
	45.7	101	3.9	3.1

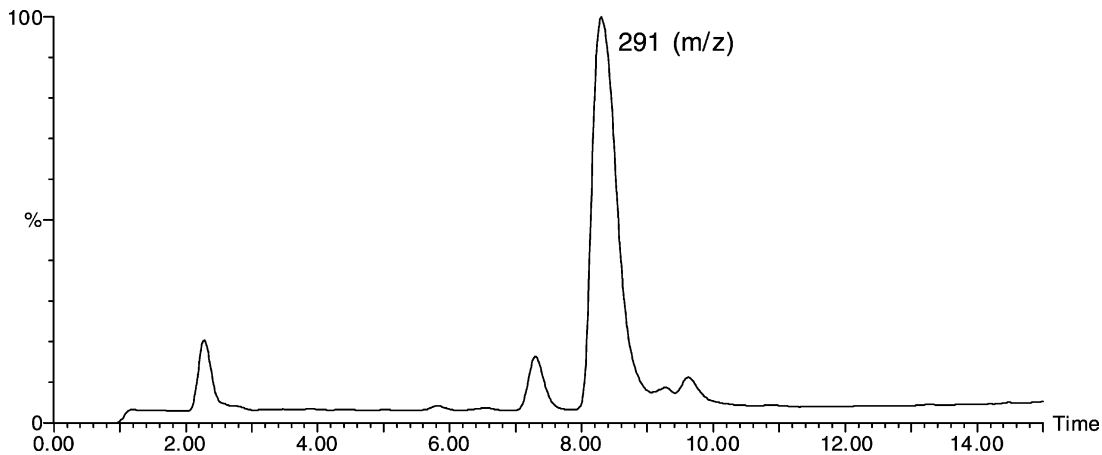


Fig. 2. Mass chromatogram (LC–MS) for the fraction of 3-OH-ropivacaine collected from LC–UV.

prepared. The ratios of peak areas of solutes and the internal standard were measured and a standard curve without the zero concentration was constructed. Calibration curves were typically described by the equation:

$$y = Ax^2 + Bx + C$$

where y is the peak-area ratio, x is the concentration of analyte and B and C are the slope and intercept, respectively, and A is the curvature. The calibration curves were weighted ($1/x$). The quality control samples (1.0 ml) were treated in the same way as the standards. Selectivity, accuracy, precision, recovery

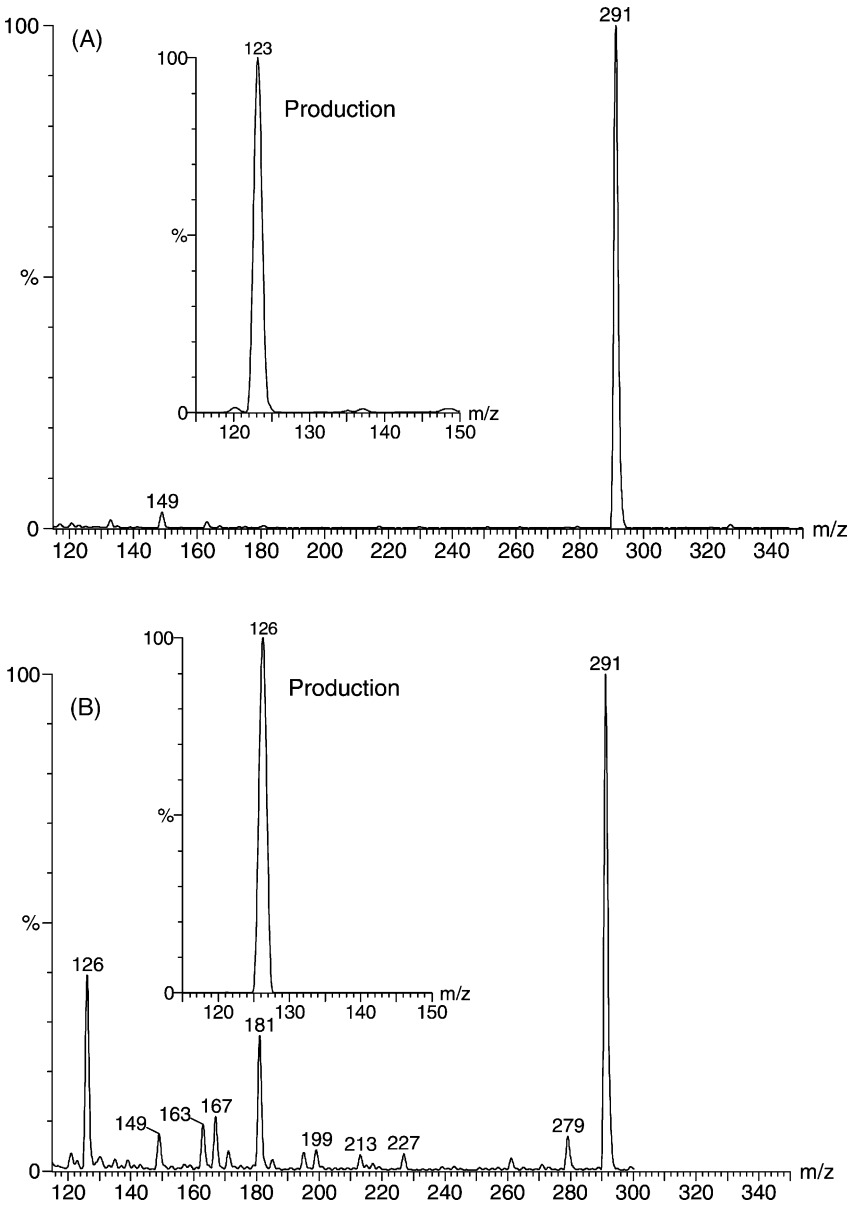


Fig. 3. Mass spectrum of precursor and product ions for trimethoprim (A) and 3-OH-ropivacaine (B).

and limit of quantification were studied according to Shah et al. [16].

The accuracy is determined as the ratio of the found and the theoretical concentration at three different concentrations. The precision is determined as coefficient of variation of the within- and between-day (intra- and inter-assay) variations at three different concentrations (Tables 1 and 2).

2.5. Urine samples

Urine samples from children treated with ropivacaine (2 mg/kg) were collected at various time points. Urine was kept at −20 °C until assay. The urine samples were thawed at room temperature. Samples were analysed either according to the present method (LC–MS–MS) or by the method described previously (LC–UV) [13,15].

3. Results and discussion

One of the challenges in bioanalysis is the analysis of samples from patients who are treated with different drugs at the same time. The more similar the structure of these drugs, the more difficult it is to separate them on chromatography systems. This problem is discussed in this paper. We have developed an accurate and simple methodology for the determination of ropivacaine and its metabolites in biological samples [15]. The approach consists of solid-phase extraction off-line with LC–UV. This method is cheap, easy to use and does not need well-trained personnel. It has been used for many hundreds of samples.

In one study for the determination of ropivacaine and its metabolites (3-OH-ropivacaine and PPX) in urine samples we found very high concentrations of 3-OH-ropivacaine only. In some urine samples the excreted amount of 3-OH-ropivacaine was twice as high as the given dose of ropivacaine. The validation showed that the methodology was accurate and the coefficient of variation was within the accepted criteria [16] for quality control samples (Table 1). The next step was to collect the fraction of 3-OH-ropivacaine peak from LC–UV and examine it by LC–MS. The analysis of the fraction showed a high concentration of 3-OH-ropivacaine (291 *m*/*z*, Fig. 2). In this case the situation was more complicated because the results from LC–MS were in agreement with LC–UV. From the clinical doctor, we got the following information: the patients in this study were treated with different drugs at the same time. These drugs

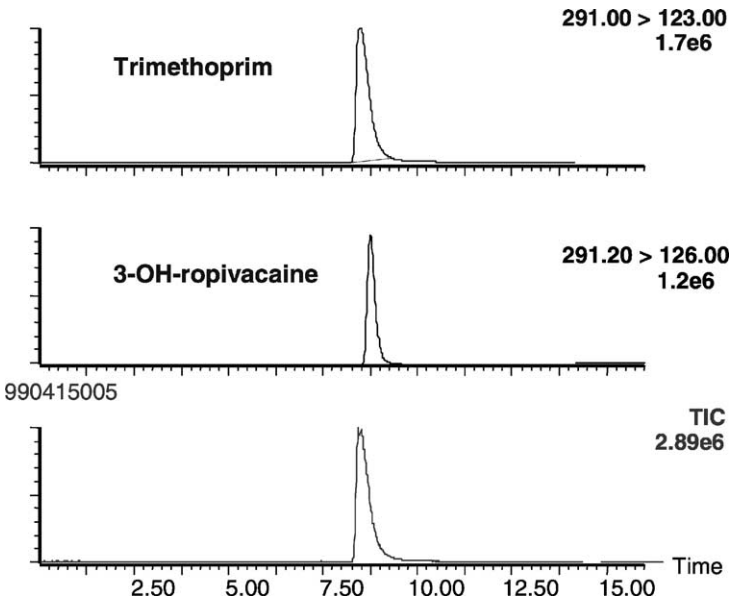


Fig. 4. Mass chromatogram of patient urine sample shows 3-OH-ropivacaine and trimethoprim by LC–MS–MS.

Table 3
The concentration of 3-OH-ropivacaine in some of the patients' urine samples using LC–UV and LC–MS–MS

Patient no.	Sample		Concentration of 3-OH-ropivacaine (μM)		Error (%)
	Start	End (h)	LC–MS–MS	LC–UV	(LC–UV/LC–MS–MS) × 100
102	2.23	8.15	47.1	94.2	200
	8.15	14.40	12.4	55.1	444
	14.40	20.23	24.9	199	799
104	3.50	9.45	150	118.6	80
	9.45	15.42	46.5	93.5	200
	15.42	21.62	31.2	175.5	560
	21.62	6.20	5.90	91.7	1550
106	0.53	6.28	19.0	78.7	414
	6.28	12.28	18.2	70.1	385
	12.28	18.87	10.9	34.2	314
108	0.38	6.03	28.3	53.8	190
	6.03	12.23	14.3	53.6	375
	12.23	18.40	6.7	117.0	1750
	18.40	24.07	2.9	139.4	4800
128	6.58	12.83	36.4	66.6	180
	12.83	18.56	24.9	69.5	280
	18.56	24.92	5.7	164.8	2890
	24.92	30.58	2.7	159.0	5990
129	4.85	11.85	59.2	76.0	128
	11.85	17.60	52.9	44.9	85
	17.60	24.85	47.1	64.1	136
	24.85	29.10	3.9	33.7	864

were paracetamol, lidocaine, fentanyl, morphine and trimethoprim.

Now either one of these drugs or one of their metabolites is similar to 3-OH-ropivacaine and has the same mass number. When we looked through the drugs, we found that trimethoprim (Fig. 1B) has the same molecular weight as 3-OH-ropivacaine (290 u). When trimethoprim was injected to the LC–UV and LC–MS systems, it gave a peak at the same retention time as 3-OH-ropivacaine. The tuning of the MS–MS system with the solutions of 3-OH-ropivacaine and trimethoprim showed that the two compounds have the same precursor ions but different product ions (Fig. 3A and B). The precursor ions were 291 and 291 and the product ions were 126 and 123 for 3-OH-ropivacaine and trimethoprim, respectively. A new method using LC–MS–MS has been set up and validated to determine 3-OH-ropivacaine. The validation showed that the method is selective, accurate and sensitive (Table 2). Fig. 4 shows the selectivity of this method: 3-OH-ropivacaine and trimethoprim could be separated in the third quadruspole by the fragments of different production ions that are generated in the collision cell. Table 3 shows the concentration of 3-OH-ropivacaine in urine samples obtained from LC–UV and LC–MS–MS analysis. In some samples when using the LC–UV method, the concentration of 3-OH-ropivacaine was higher by a factor of 50 than the true value.

4. Conclusion

This study illustrates the necessity of using the selective MS–MS technique for analysis of biological samples from patients who are treated concomitantly with several drugs. Although the validation data of the LC–UV method using spiked urine samples were excellent within the acceptance criteria, the method gave incorrect/unbelievable results with the samples

obtained. Reanalysis of the fraction containing 3-OH-ropivacaine with the selective LC–MS–MS technique showed co-elution with a compound with the same precursor ion but a different product ion. This means that the LC–MS technique does not have the requisite selectivity for patient samples either.

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