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Validation of a method for quantification of ketobemidone in human plasma with liquid chromatography–tandem mass spectrometry

Matilda Lampinen[a], Ulf Bondesson[a,b], Elisabeth Fredriksson[b], Mikael Hedeland[a,b,*]

[a] *Division of Analytical Pharmaceutical Chemistry, Uppsala University, Biomedical Centre, Box 574, SE-751 23 Uppsala, Sweden*
[b] *Department of Chemistry, Section of Drug Analysis, National Veterinary Institute (SVA), SE-751 89 Uppsala, Sweden*

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Abstract

A liquid chromatography tandem mass spectrometry (LC–MS–MS) method for determination of the analgesic aminophenol ketobemidone in human plasma is presented. Two preparation methods for plasma samples containing ketobemidone were compared, liquid–liquid extraction (LLE) and solid-phase extraction (SPE). Both methods showed good precision ($n=10$), 1.7% and 2.9%, respectively (0.04 μM) and 1.1% and 2.5%, respectively (0.14 μM). The accuracy was 98% and 103%, respectively (0.04 μM) and 105% and 99%, respectively (0.14 μM). Ketobemidone could be quantified at 0.43 n*M*, with a relative standard deviation of 17.5% ($n=19$) using LLE and 18.6% ($n=10$) using SPE. This level was an order of magnitude lower than earlier reported quantification limits. Quantitative data from plasma samples analyzed with LC–MS–MS were in good agreement with those obtained by gas chromatography with chemical ionization mass spectrometry (GC-CI/MS). This indicates that LC–MS–MS is a good alternative method to GC–MS as it is more sensitive and time-consuming derivatization can be avoided.

Keywords: Ketobemidone

1. Introduction

Ketobemidone is a narcotic analgesic drug which is given to patients in severe pain. It is an amino phenolic compound with ampholytic character, see Fig. 1. The metabolism of ketobemidone has been studied by analysis of human urine using liquid–liquid extraction (LLE), derivatization and gas chromatography mass spectrometry with electron ionization (GC-EI/MS) [1]. In a later study, five phase I metabolites of ketobemidone were identified in urine samples, using solid-phase extraction (SPE) together with LC–MS–MS [2]. Furthermore, glucuronic acid conjugates of ketobemidone and two of its phase I metabolites have been identified in urine with accurate mass determination using time-of-flight mass spectrometry [3].

A few methods for quantification of ketobemidone and its metabolites in human plasma and urine have also been published. These were based on LLE and derivatization together with GC-EI/MS [1,4,5], and SPE together with LC-single quadrupole-MS [6,7]. The advantage of using LC instead of GC is that time-consuming derivatization can be avoided. Fur-

*Corresponding author. Tel.: +46-18-674-209; fax: +46-18-674-099.
E-mail address: mikael.hedeland@sva.se (M. Hedeland).

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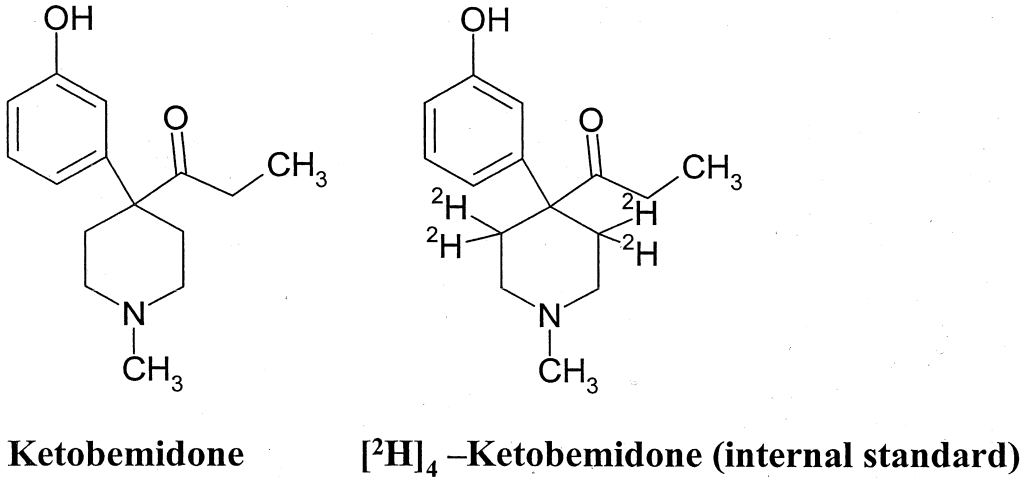

Fig. 1. Structures of ketobemidone and the internal standard.

thermore, the use of SPE instead of LLE provides a sample preparation method that easily could be automated. A higher sensitivity and selectivity could probably be obtained by using LC–MS–MS compared to previously published methods based on LC-single quadrupole-MS. This could be an advantage to avoid extensive extrapolation in pharmacokinetic studies or when low sample volumes are available, e.g. studies on children.

The aim of this study was to validate an LC–MS–MS method for quantification of ketobemidone in human plasma and to compare its performance with those published earlier.

2. Experimental

2.1. Chemicals and materials

Ketobemidone hydrochloride was obtained from Pharmacia (Uppsala, Sweden) and [$^2H_4$]-ketobemidone hydrochloride (internal standard) was synthesized according to a previously published method [8], see structures in Fig. 1. The water was purified using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). All other chemicals were of analytical reagent grade and used without further purification. Human blank plasma was obtained from the Academic Hospital (Uppsala, Sweden). Plasma samples were collected from patients given Ketogan Novum® (Pharmacia Sverige, Sweden) containing ketobemidone hydrochloride 5 mg/ml using an intravenous patient-controlled pump. The drug was given to five patients as pain control after coronary bypass surgery. Three plasma samples were collected from each patient at different times. The study was started after approval by the ethics committee and informed patient consent.

2.2. Instrumental procedures

2.2.1. GC–MS

A HP 5890 gas chromatograph (Hewlett-Packard, Waldbrunn, Germany), with a 5% phenyl/methyl silicone-capillary column (25 m×0.32 μm) film thickness 17 μm (Hewlett-Packard) was used. The mass spectrometer was an SSQ model 710 single quadrupole (Finnigan, San Jose, CA, USA). For instrument control, a computer with the software ICIS 8.3.0 (Finnigan) was used. The temperature settings for the gas chromatograph were: injector 185 °C, the oven was programmed to 70 °C the first minute and then the temperature was gradually increased with 30 °C/min to 290 °C and the transfer line was set at 290 °C. The mass spectrometric conditions were: positive chemical ionization with ammonia as reagent gas. The filament was set at 400 mA and the multiple-ion detector at 1900 V.

The mass analyzer was programmed for SIM

(selected ion monitoring), to record the $[M+H]^+$ ions m/z 394 and m/z 398 (ketobemidone pentafluoropropionic acid anhydride (PFPA) derivative and $[^2H_4]$-ketobemidone PFPA derivative, respectively).

2.2.2. LC–MS–MS

An Agilent 1100 series liquid chromatograph (Agilent Technologies, Waldbrunn, Germany) with a binary pump, degasser and an autosampler, was used. The mobile phase was 40% methanol in 0.1 *M* acetic acid in Milli-Q-water. The flow-rate was 0.2 ml/min and the injection volume was 5.0 μl. A Synergi Polar RP column (Phenomenex, Torrance, CA, USA) with a particle diameter of 4 μm and dimensions of 150 mm×2.00 mm (length×I.D.) was used.

The HPLC system was coupled to a Quattro LC (Micromass, Manchester, UK) triple quadrupole mass spectrometer equipped with an electrospray interface (ESI). The controller for these instruments was a PC with the software MassLynx v. 3.3. This program was also used for data acquisition, processing and integration of peaks. The integration was performed with a smoothing method of mean and the number of smoothings was two.

The mass spectrometric parameters were tuned for optimization of the sensitivity. This was done with direct infusion of a ketobemidone standard solution with a syringe pump at 5 μl/min through a connecting T where it was mixed with the LC mobile phase (flow-rate 0.2 ml/min). The parameters for the ESI were set as follows; capillary voltage 1.00 kV, cone 36 V, extractor 8 V and RF lens 0.80 V. The desolvation temperature was 350 °C and the source block temperature 120 °C. The nebulizer gas flow was 104 l/h and the desolvation gas flow was 904 l/h. For MS–MS analysis the collision cell was filled with argon at a pressure of 5.3×10^{-4} mbar and the collision energy was set at 28.0 eV. The instrument was run in the selected reaction monitoring (SRM) mode, where the first quadrupole was switching between m/z 248 (ketobemidone $[M+H]^+$) and m/z 252 ($[^2H_4]$-ketobemidone $[M+H]^+$), and the second one was switched between m/z 190 and m/z 194, which are daughter fragment ions from ketobemidone and $[^2H_4]$-ketobemidone, respectively.

2.3. Sample preparation

2.3.1. Standard curve

Ketobemidone hydrochloride and $[^2H_4]$-ketobemidone hydrochloride (internal standard) were weighed and dissolved in water. The stock solution of ketobemidone was used to prepare standards at different concentrations in water for the standard curve. To 1.0 ml of blank human plasma 100 μl of the respective standard solution were added. The standard curves were constructed with the peak area ratio (ketobemidone/internal standard) as a function of ketobemidone concentration. Four different standard curve intervals were used (see Table 1).

2.3.2. Patient plasma samples

Patient plasma samples were prepared together with the standard samples. Three samples from each patient gave a total of 15 plasma samples.

2.3.3. Liquid–liquid extraction (LLE)

The liquid–liquid extraction method was slightly modified from an earlier study [4].

To 1.0 ml plasma (standard, patient or control

Table 1
Validation data

		n	Ketobemidone (n*M*)	Standard curve concentration interval (n*M*)	Correlation (R^2)	Precision (RSD%)	Accuracy (%)
LLE	QC-1	10	43	3.80–384	0.9982	1.7	97.9
	QC-2	10	137	3.80–384	0.9996	1.1	105.4
	QC-3	19	0.43	0.40–19.4	0.9988	17.5	132.6
GC–MS	QC-1	10	43	3.80–384	0.9918	16.6	101.1
SPE	QC-1	10	40	1.17–241	0.9997	2.9	103.5
	QC-2	10	139	1.17–241	0.9997	2.5	98.9
	QC-3	10	0.43	0.12–23.4	0.9995	18.3	121.9

sample), 100 μl internal standard (final concentration in plasma 0.10 μ*M*) and 5.0 ml toluene–2-butanol (9:1) were added together with 1.0 ml saturated carbonate buffer pH 9.35. The samples were shaken for 10 min and then centrifuged for 10 min at 3500 rev./min. The organic phase was transferred to new tubes containing 1.0 ml 0.05 *M* H_2SO_4. The samples were shaken and centrifuged as before. Dichloromethane–2-butanol (8:2) (4.0 ml) and 1.0 ml saturated carbonate buffer (pH 9.35) were added to the water phase. After a 10-min extraction centrifugation, the organic phase was poured into new tubes and evaporated to dryness. For the LC–MS–MS analysis the residues were dissolved in 50 μl 0.1 *M* acetic acid in water, vortex shaken and transferred to microvials.

The samples prepared for GC–MS analysis were prepared as above except for the last step, where instead of redissolution in acetic acid, the samples were derivatized with 50 μl PFPA in an oven (80 °C) for 20 min. The samples were then evaporated and the residues were dissolved in 50 μl toluene and transferred to microvials for analysis.

2.3.4. SPE

To 1.0 ml plasma 100 μl standard and 100 μl internal standard (final concentration in plasma 0.10 μ*M*) were added. For precipitation of proteins, 1.0 ml 10% trichloroacetic acid (TCA) was added and the sample was vortex shaken for 1 min and centrifuged at 3500 rev./min for 10 min.

The SPE column, SPEC MP 3 (mixed-mode slightly polar cation-exchange) microcolumn discs 15 mg (Division of Ansys, Irvine, CA, USA), was coupled to a vacuum manifold and activated with 1.0 ml methanol and 1.0 ml water. The supernatant from the sample was decanted onto the column, which was subsequently washed with 1.0 ml water and dried with vacuum for 10 min. The sample was eluted with 1.0 ml 1% ammonia in dichloromethane. The organic phase was evaporated and the residue was dissolved in 50 μl 0.1 *M* acetic acid in water, vortex shaken and transferred to microvials prior to LC–MS–MS analysis.

2.3.5. Validation

The method was validated for linearity, precision, accuracy and sensitivity by analysis of human plasma spiked at three levels (QC-1, QC-2 and QC-3; Table 1). The replicates were prepared as follows; 1.1 ml standard was added to 11.0 ml human plasma and 10 replicates each containing 1.0 ml were taken from the spiked plasma. This procedure was applied for every QC sample level, so that any possible concentration difference caused by standard addition was minimized.

3. Results and discussion

3.1. Method development

In the present study, a method for quantification of ketobemidone in human plasma with two different sample pretreatment techniques has been validated. LLE [4] and SPE [7] of ketobemidone from plasma as sample pretreatment methods for GC-EI/MS and LC-single quadrupole-MS, respectively, have been published earlier. This paper describes an evaluation and comparison between LLE and SPE in combination with the more sensitive and selective SRM mode in LC–MS–MS. During the method development, one has to consider issues such as purity of the extracts, selectivity, linearity, precision, accuracy and sensitivity of the LC–MS–MS method, as well as ease of method operation.

In the LLE experiments, an addition of 2-butanol was used in the organic phase in order to improve its hydrogen bonding ability [4]. The aqueous phase pH was chosen to be 9.35, which was within the earlier stated optimal range [4]. A back extraction at low pH was performed to remove acidic and neutral substances that could interfere with the analysis.

Mixed-mode SPE methods for the extraction of morphine has earlier been described by other authors [9,10]. As ketobemidone is also an amino phenol, this kind of SPE was thus chosen in this study as well. Precipitation of plasma proteins was necessary in order to avoid clogging of the SPE columns. It was performed with TCA, as an addition of organic solvents would increase the elution strength of the sample medium and thus result in a lower recovery in the subsequent SPE step. The acidification of the sample, yielding a positive net charge of ketobemidone, was not a problem, as the solid-phase consisted of a mixture of cation-exchangers and a

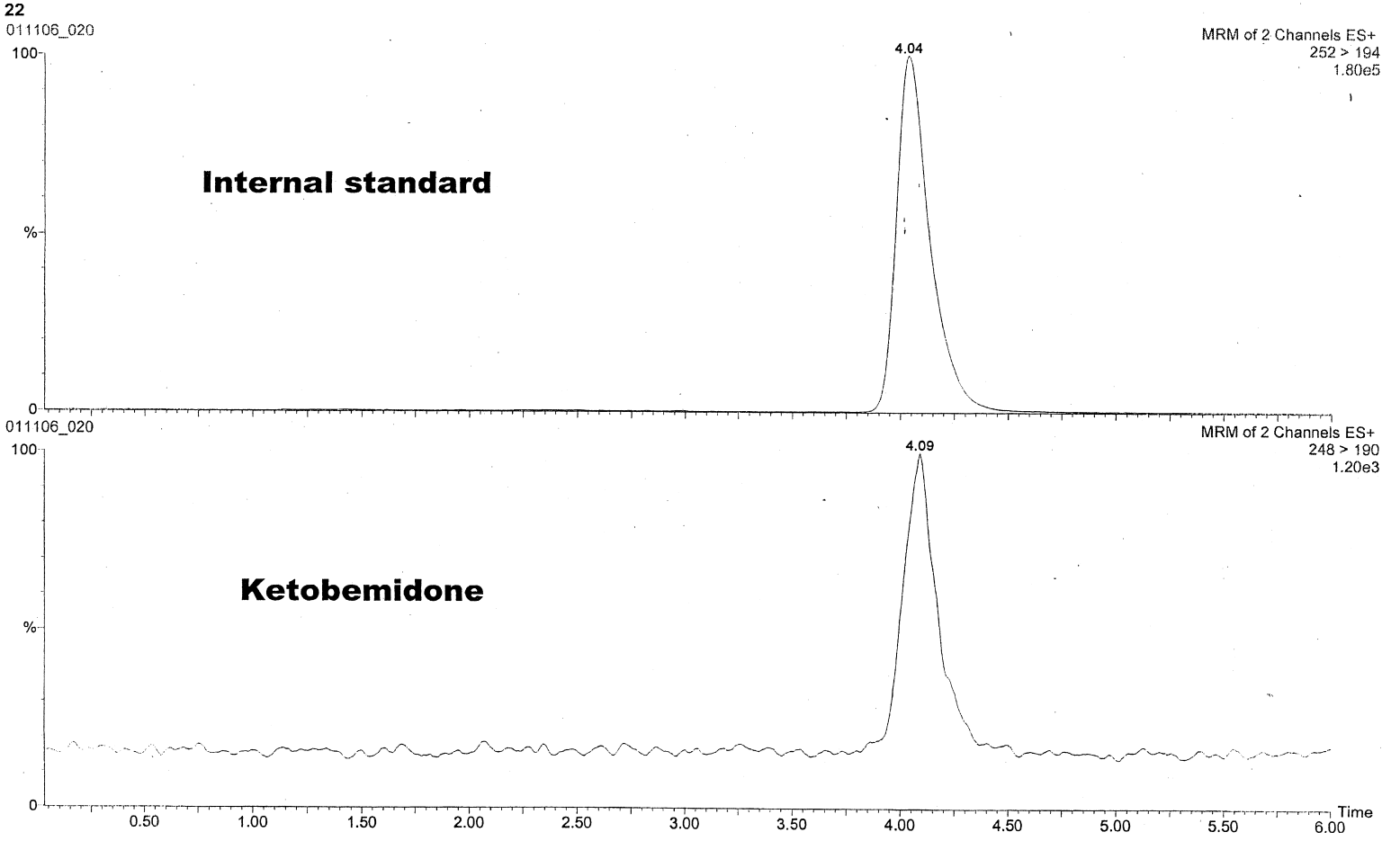

Fig. 2. Chromatogram of a typical low control sample (0.43 n*M* ketobemidone in plasma).

slightly polar surface. The elution was carried out at alkaline conditions with a mixture of ammonia and dichloromethane, as it had the combined effect of decreasing the electrostatic attractions and hydrophobic interactions between ketobemidone and the solid-phase.

Selected reaction monitoring (SRM) was chosen for data acquisition as this is usually the most selective and sensitive mode for quantification using a triple quadrupole mass spectrometer. The collision gas (argon) pressure and collision energy were optimized in order to obtain maximal transformation of the respective parent ion to one single daughter ion and to avoid further fragmentation. This would give the highest possible sensitivity for quantification.

Isocratic LC conditions with a simple and volatile mobile phase consisting of water, methanol and acetic acid, was proven to give sufficiently reproducible results (see Table 1). This was advantageous compared with gradient elution, as long equilibration times between injections could be avoided. When using LC coupled to such a selective detection system as a tandem mass spectrometer running in the SRM mode, insufficient chromatographic separation was not a problem.

Ketobemidone deuterated in four positions ([$^2H_4$]-ketobemidone) was chosen as internal standard for relative peak area standardization. This solute was considered to be the optimal internal standard, as it has almost identical physico-chemical properties as the sample, while it was still separately detected by the mass spectrometer. Ketobemidone and [$^2H_4$]-ketobemidone could be detected within 4.13 min (Fig. 2).

3.2. Validation

3.2.1. Linearity

All standard curves for LC–MS–MS showed good linearity ($r^2 \geq 0.998$). The standard curve intervals and correlation coefficients are shown in Table 1.

3.2.2. Precision

The precisions for the two control sample levels QC-1 and QC-2 were excellent for LLE and SPE (Table 1). The QC-1 samples worked up with either LLE or SPE gave an RSD equal to or lower compared to an earlier published study (in Ref. [7] RSD = 2.8% at 38.9 n*M*). The QC-3 sample at 0.43 n*M* gave a precision of 17–18% (Table 1, Fig. 2). Thus, ketobemidone could with this method be quantified at a level an order of magnitude lower than in the earlier published ones (in Ref. [7] C.V. < 25% at 3 nM; in Ref. [4] LOQ 2 ng/ml (~8 nM)). The RSD for the QC-1 analyzed with GC-CI/MS was about 10 times higher than the results from LLE with LC–MS–MS, and about five times higher than the results from the SPE with LC–MS–MS. Earlier results with GC-EI/MS gave an RSD of 4% at 5 ng/ml (~20 n*M*) [4] and 8% at 10 ng/ml (~40 n*M*) [5]. Thus, the LC–MS–MS method presented in this study provides a more sensitive determination and also a higher precision than earlier published methods based on LC-single quadrupole-MS or GC–MS.

3.2.3. Accuracy

The accuracies for the QC-1 and QC-2 control samples were close to 100% (Table 1). Unfortunately no values of accuracy were found in earlier plasma studies to compare with [4,5,7]. The QC-3 control sample accuracy was slightly higher than 100%, but that was probably due to chromatographic peak splitting at the lowest concentration. This effect was investigated by full daughter ion scan. The spectra in the front and tail of the split peak were

Table 2
Analysis of patient samples with GC-CI/MS and LC–MS–MS

Sample	GC–MS (nM)	LC–MS–MS (nM)	Difference (%)
A.1	28.7	33.2	−15.7
A.2	34.8	39.2	−12.6
A.3	27.9	28.3	−1.4
B.1	110	89.4	18.7
B.2	87.7	112.8	−28.6
B.3	76.4	69.5	9.0
C.1	127.4	135.4	−6.3
C.2	98.7	105.9	−7.3
C.3	65.5	66.3	−1.2
D.1	101.5	123.7	−21.9
D.2	123.3	114	7.5
D.3	118.5	132.2	−11.6
E.1	219.9	241.8	−10.0
E.2	187.2	208.2	−11.2
E.3	122.5	125.7	−2.6

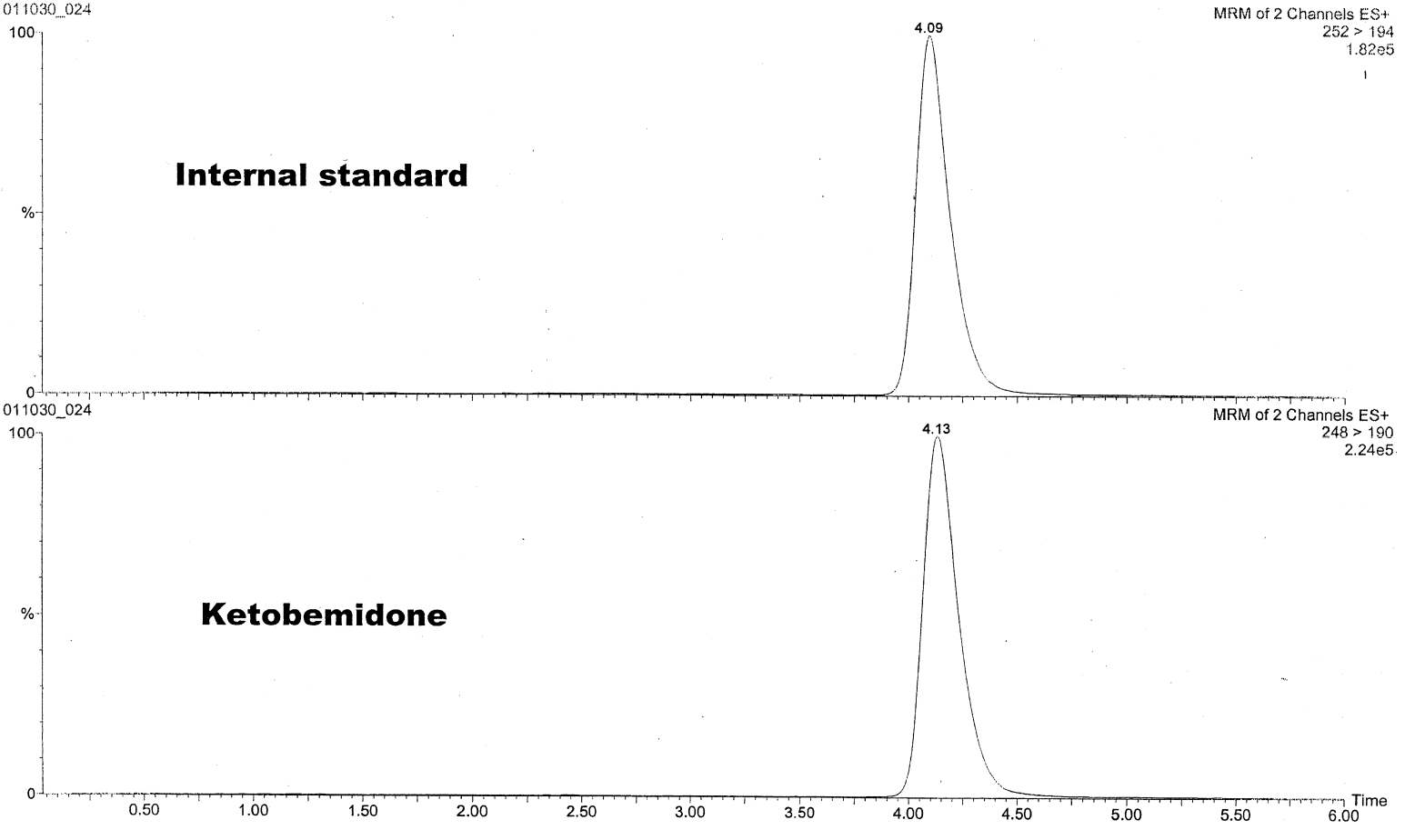

Fig. 3. Chromatogram of a typical patient sample with a determined concentration of 112.8 n*M* ketobemidone.

identical, indicating that the observed phenomenon was an unexplained chromatographic effect rather than a chemical interference (results not shown).

3.3. Patient samples

Table 2 shows a comparison of the results from quantitative determinations of ketobemidone with LC–MS–MS and GC-CI/MS in patient plasma samples. The determined sample concentrations of ketobemidone from both methods were in the same range. For 11 out of 15 compared samples, the difference is within ±15% and in one case, just above 15%. The accuracy of an analytical method should be within ±15% of the true value [11]. The difference between the two methods was thus in the majority of cases within these limits. The samples were prepared at two different occasions for the different methods and they were frozen 4 months in between. The majority of the plasma samples had a higher determined concentration when analyzed with LC–MS–MS compared to GC-CI/MS. One notable exception was the sample denoted B.1, which had a significantly lower determined value with LC–MS–MS. The reason for this has not been elucidated. The samples could have been affected during storage. However, the high RSD value for the QC-sample determined with the GC-CI/MS method showed that the uncertainty of this method was higher (Table 1). A typical chromatogram of a patient plasma sample is shown in Fig. 3.

4. Conclusions

An LC–MS–MS method has been validated for quantitative determination of ketobemidone in human plasma. The advantage of the present LC–MS–MS method compared with GC–MS is that no derivatization is necessary before the analysis. Sample preparation with SPE gave almost as good precision as with LLE. The option to use SPE instead of LLE provides an even more simplified sample preparation. This method based on LC–MS–MS made it possible to determine ketobemidone in plasma concentrations down to 0.43 n*M* with a precision better than 18.6%. This level is an order of magnitude lower compared with the earlier published methods based on LC-single quadrupole-MS or GC–MS. This could be important in determinations of low concentration samples, e.g. samples from the elimination phase in a pharmacokinetic study.

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