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Quantitative determination of piritramide in human serum applying liquid chromatography–two-stage mass spectrometry

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Abstract

A method for the determination of the synthetic narcotic analgesic piritramide in human serum utilizing high-performance liquid chromatography with atmospheric pressure chemical ionization two-stage mass spectrometry (HPLC–APCI-MS–MS) is presented. Pipamperone is used as the internal standard. Serum samples are prepared by liquid–liquid extraction under basic conditions with 1-chlorobutane. The chromatographic separation is achieved on an RP-18 stationary phase applying gradient elution with methanol–0.02% trifluoroacetic acid in water. Detection is carried out in the MS–MS single reaction monitoring mode of the ion-trap mass spectrometer. The limit of detection is 0.3 ng/ml and the calibration covers the range of 1–80 ng/ml. The intra-day RSDs are 6.1 to 7.3% over the calibration range, whereas the inter-day RSDs are 9.6 to 12.8%.

Keywords: Piritramide

1. Introduction

Piritramide (Fig. 1) is a synthetic narcotic analgesic indicated primarily for the treatment of postoperative pain. Commonly, it is applied with good success via a portable pump in a regime called patient controlled analgesia (PCA) [1,2]. Its steady-state concentration necessary to produce 50% of maximum analgesia is 12.1 ng/ml (2.9 to 29.8 ng/ml) [3]. In comparison to other opioid analgesia, it has a quite slow onset of action (10 to 60 min) [4,5] and a long terminal elimination half live of about 8 h, with considerable variability with respect to the age of the patients [6]. It therefore has to be dosed with care during long-term treatment to avoid accumulation that may lead to adverse effects [7] and the determination of serum concentrations is very helpful to manage cases of delayed elimination or unexpected side effects.

Analytical assays for piritramide in human samples are very scarce. A high-performance liquid chromatography (HPLC) procedure [8] with liquid–liquid extraction is not sensitive enough (0.5–2.5 μg/ml) to be utilized on real patient samples. A method for gas chromatography with nitrogen–phosphorus sensitive detection gives a much better sensitivity (limit of detection, LOD 1–2 ng/ml) [9]. Occasionally, piritramide serves as internal standard (I.S.) in assays for other substances (e.g., Ref. [10]),

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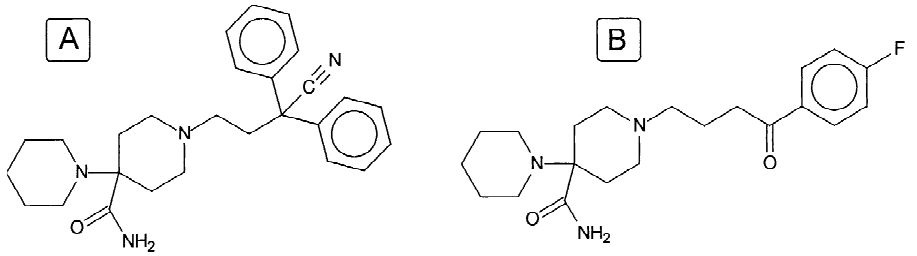

Fig. 1. Chemical structures of piritramide (A) and the I.S. pipamperone (B).

but these assays can not be readily adopted for the determination of piritramide itself.

In this paper, we describe the very sensitive determination of piritramide in human serum applying high-performance liquid chromatography–atmospheric pressure chemical ionization two-stage mass spectrometry (HPLC–APCI-MS–MS). Due to the selectivity achieved with this detection method, a relatively easy sample preparation with a one-step liquid–liquid extraction and a fast HPLC method is sufficient to detect piritramide without interferences in concentrations as low as 0.3 ng/ml.

2. Experimental

2.1. Instrumentation

The HPLC part of the analytical system consists of an Agilent 1100 system (Waldbronn, Germany) comprising a degasser, a binary pump, an autosampler and a thermostatted column compartment, controlled by an Agilent 1100 control module. The chromatographic separation of the analytes took place in a Merck Supersphere 100 RP-18 endcapped 125×4 mm column equipped with a 4×4 mm guard column filled with the same material. The analytes were detected by a ThermoFinnigan LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an APCI source. Data were collected and analyzed by the ThermoFinnigan Xcalibur software package, revision 1.1.

2.2. Chemicals

Piritramide and the I.S. pipamperone were obtained as pure substances from Janssen-Cilag (Neuss, Germany). Trifluoroacetic acid was purchased from Riedel-de Haën (Seelze, Germany), methanol in gradient-grade from Merck (Darmstadt, Germany). Ultra pure water was produced in our laboratory by a Barnstead EASYpure UV system (Werner, Leverkusen, Germany). All other chemicals were of analytical grade or better.

2.3. Sample collection

Blood samples of about 5 ml were drawn from the cubital vein into glass containers without additives. Serum was obtained by separation of the blood cells by centrifugation at 2400 *g* for 10 min. Serum samples were either analyzed immediately or were frozen at −20 °C until analysis.

2.4. Calibration samples

A stock solution of piritramide in water was prepared by dissolving 10 mg piritramide in about 5 ml 0.02 *M* HCl. Subsequently the volume was added up to 100 ml with water to yield a concentration of 100 μg/ml. This stock solution was diluted by a factor of 100 with water to get the working standard solution. Adding up 1, 2, 5, 10, 20, 40, 60 and 80 μl of the working standard solution with blank human serum to 1 ml produced eight calibration levels in the concentration range of 1 to 80 ng/ml.

2.5. Quality control samples

Quality control samples were prepared from drug free human serum at four concentration levels (1, 5, 20 and 80 ng/ml). A sufficient number of 1-ml samples were portioned and frozen at −20 °C until analysis. To exclude any sample alteration under

these conditions, quality control samples were tested against freshly prepared ones and spiked frozen pool serum.

2.6. Sample preparation

To 1 ml serum, 100 μl of the I.S. solution (1 μg/ml pipamperone in water) and 100 μl of 1 *M* NaOH were added. This mixture was extracted twice with 3 ml 1-chlorobutane for 20 min on a roller-shaker. Phase separation was achieved by centrifugation at 4000 *g* for 5 min. The combined organic phases were evaporated in vacuum. The residue was dissolved in 50 μl methanol and 100 μl trifluoroacetic acid 0.02% in water. After intense shaking for 10 s the sample was forwarded to the HPLC system.

2.7. Determination of the extraction recovery

The extraction yield was determined by comparing extracts from spiked serum with extracts from blank serum, spiked with the analytes after the extraction in concentrations corresponding to 100% extraction yield. The samples number *n* for each the extracted samples as well as the 100% samples was 6.

2.8. Chromatographic conditions and MS detector settings

From the extracted sample, 50 μl was injected into the HPLC system. The chromatographic separation of the analytes was accomplished by gradient elution starting with trifluoroacetic acid 0.02% in water–methanol (55:45) at a flow-rate of 0.8 ml/min. The percentage of methanol was raised in the first 0.5 min to 70% and was held constant for the rest of the run. After the end of the run at 10 min a column re-equilibration time of 3.5 min before the next injection was necessary. With a column temperature of 35 °C, the retention times of the I.S. pipamperone and piritramide were 3.7 and 5.6 min, respectively.

The settings for the APCI ion source were as follows: nebulizer temperature and the capillary temperature were set to 450 and 200 °C, respectively. The capillary voltage was set to 26 V and a discharge current of 5 μA was applied. The sheath gas flow-rate was set to 40 units (about 0.6 l/min), the auxiliary gas remained turned off. A divert valve directs the HPLC-flow in the first 2.5 min of the chromatographic run to the waste container and afterwards to the ion source. The APCI source was working in the ''positive mode'', producing positive charged ions in the form of $[H^+]$ adduct ions. From the ions generated in this way, those with mass–charge ratios of 376 m/z and 431 m/z were trapped, representing the I.S. pipamperone and piritramide, respectively. These trapped ions were further fragmented by collision induced dissociation. The ion 376 m/z dissociated at a relative collision energy of 26% [this corresponds to 1.3 V peak-to-peak resonance excitation radiofrequency (RF) voltage] into its main fragments with relative masses of 291 and 331 m/z. The ion 431 m/z dissociated at a relative collision energy of 26% to 346 and 386 m/z. The chromatographic traces of the precursor ions and their respective product ions were monitored in the selected reaction monitoring mode of the mass spectrometer.

3. Results and discussion

3.1. Extraction efficiency

The extraction solvent 1-chlorobutane was selected for producing high recoveries and clean extracts. Other solvents tested (toluene, hexane/amyl alcohol, ethyl acetate) proved to be less efficient or selective. The extraction recovery was determined by comparing the peak areas in extracted samples with extracts of blank serum spiked after the extraction with the analytes. This somewhat complicated procedure is necessary to exclude matrix effects of the serum extracts on the response of the analytes. The extraction recovery was 85±8.8% for piritramide and 110±8.1% for the I.S. pipamperone.

3.2. Chromatography and mass spectrometry

In Fig. 2, some typical chromatograms from serum samples obtained with the described method are depicted. No interferences from endogenous substances or, in the case of the patient's sample, from metabolites of piritramide are observed. However, some tailing of the peaks is obvious. This is due to the unbuffered mobile phase of the chromatographic

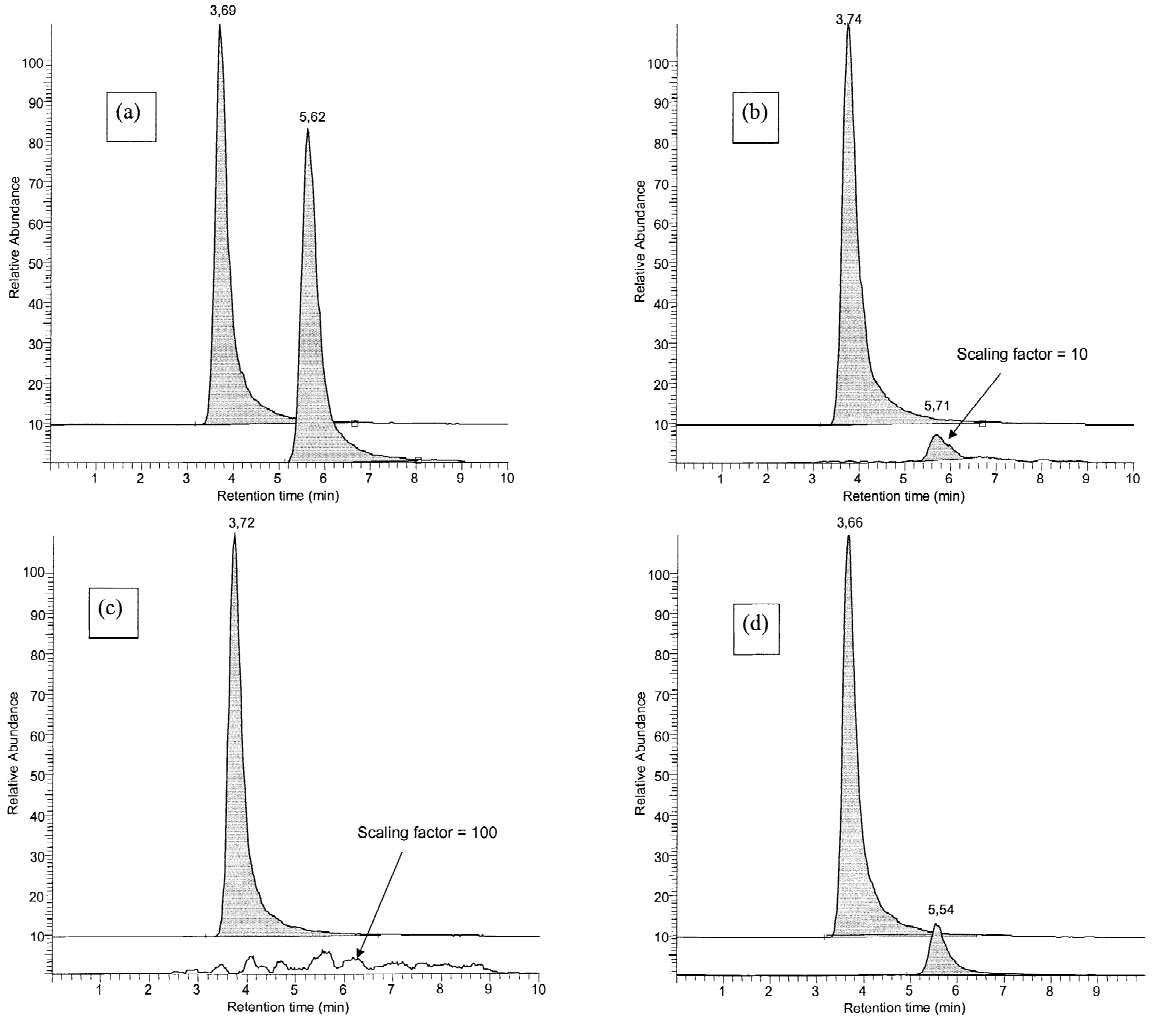

Fig. 2. Typical chromatograms obtained with the described method. The upper trace is the sum of the product ions 291+331 m/z, representing the I.S. pipamperone. The lower trace is the sum of the product ions 346+386 m/z, representing piritramide. (a) Serum sample spiked with 80 ng/ml piritramide, (b) serum sample spiked with 1 ng/ml piritramide, (c) blank human serum sample, (d) patient serum sample containing 14.2 ng/ml piritramide.

system, which in turn is obligatory to achieve good ionization efficiency in the APCI ion source. The use of methanol instead of the more common acetonitrile as organic modifier and the sharp step in the gradient program of the mobile phase serves to refocus the peaks and to keep tailing at bay. Possibly, better peak shapes may be obtained with columns especially designed for the separation of compounds with amino moieties, but they are much more expensive than the very robust and ready-off-the-shelf column utilized in this separation. Nevertheless, in spite of the tailing of the peaks, the sensitivity is sufficient to determine concentrations as low as 1 ng/ml with good precision and accuracy (see Section 3.4).

The ionization in the APCI ion source leads exclusively to the quasimolecular ions $[M+H]^+$. No sodium adducts or fragments of the analytes are observed. The collision induced fragmentation of these quasimolecular ions lead to the main fragments of 346 and 386 m/z in the case of piritramide and to 291 and 331 m/z in the case of the I.S. pipamperone. These fragment ions result from the cleavage of the

4-piperidino-carboxamide bond and the 4-piperidino-piperidyl bond in both piritramide and pipamperone. To enhance the S/N ratio and to lower the SD of the peak quantification, both ion traces referring to each analyte were summed up in the quantification processing.

Ion suppression by matrix components is a major problem in quantitative LC–MS. To prove for the absence of such effects, a diluted solution of the analytes was continually infused into the effluent of the LC via a t-union, while an extract of blank plasma is chromatographed. If over the runtime of the chromatogram any ion-suppressing substance elute, it will be detected by a decay in the signal strength of the infused analytes. Ideally, no such suppression of the continuous signal of the analytes should arise over the runtime. In Fig. 3, the result of such an experiment is depicted. As can be seen, a strong ion suppression, caused by non-retained serum constituents still present in the extracts, took place in the first 2 min after the column dead time. However, in the retention time window of the analytes no ion suppression was observed and therefore the quantification is not compromised.

3.3. Calibration and limits of detection and determination

The calibration range (1–80 ng/ml) covers therapeutic concentrations of piritramide as well as such with toxicological relevance. The calibration function (concentration vs. area piritramide/area I.S.) is linear and crosses the origin with no significant deviation. As it is quite common for such large calibration ranges, the variances of the responses of piritramide increase proportionally with the re-

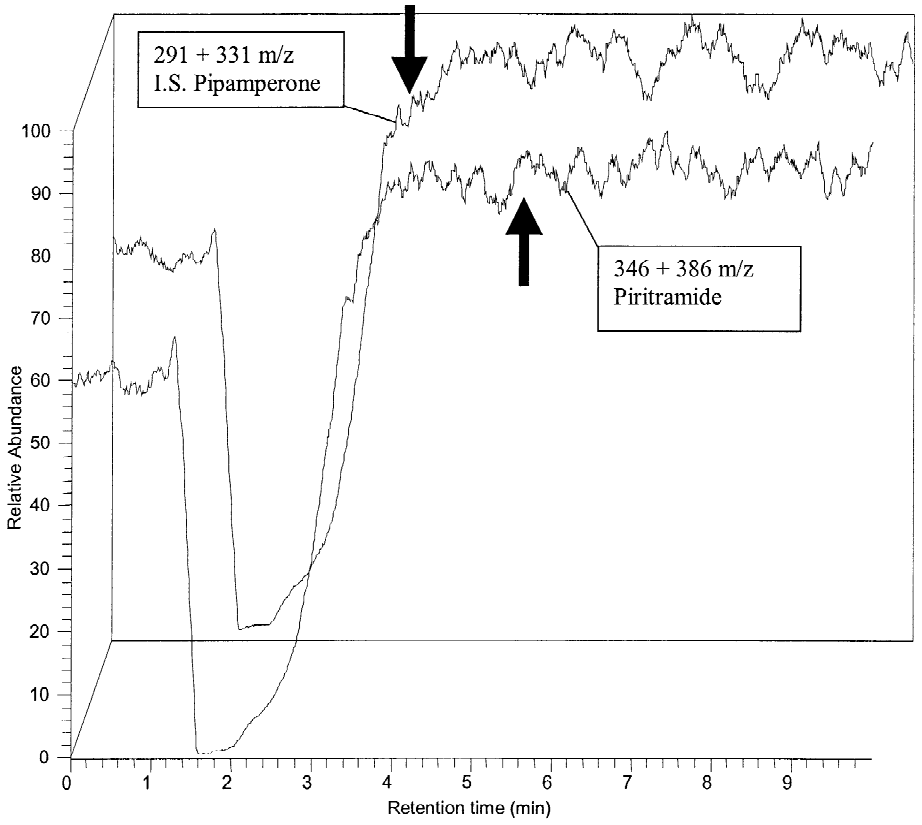

Fig. 3. Ion suppression plot (details see text) of the sum of the product ions 291+331 m/z, representing the I.S. pipamperone and the sum of the product ions 346+386 m/z, representing piritramide. The arrows indicate the retention times of the analytes in a chromatogram.

sponses itself. To pay respect to this fact, a weighted (1/response) least square function is applied. The slope of the calibration function derived from this calculation is 0.01092 ± 0.000988 ($n=6$).

The LOD of piritramid is 0.3 ng/ml, where the S/N ratio is 3. The limit of determination is 1 ng/ml (three times the LOD), with still good precision and accuracy results (see. Section 3.4).

3.4. Precision and accuracy

The validation data regarding intra-day as well as inter-day precision and accuracy are summarized in Tables 1 and 2, respectively. The inter-day precision and accuracy data were derived from the results of the quality control samples used in normal sequences. All precision and accuracy results are satisfying the requirements for a validated method [11].

3.5. Stability of the samples against freeze–thaw cycles

The overall stability of the quality control samples was proven against the freshly prepared calibration samples and show good accuracy (Table 1). To rule out any low-temperature precipitation or absorption of the analytes, responses of the quality control samples were compared to spiked pool serum after a freeze–thaw cycle, from which 1-ml portions were drawn. No significant differences ($P=0.29$, $n=6$) between spiked pool serum and the quality control samples were observed.

4. Conclusion

The described method is robust, easy and fast. It is capable for the determination of piritramide in human serum in therapeutic as well as in toxicological relevant concentrations. Due to the very selective and sensitive MS detection only a simple one-step liquid–liquid extraction is necessary to achieve chromatograms free of interferences and a limit of detection of 0.3 ng/ml. The method has been successfully applied to patient samples (example depicted in Fig. 2d) in support of therapeutic as well as forensic cases.

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Table 1
Intra-day precision and accuracy

Concentration level (ng/ml)	Measured concentration (average) (ng/ml)	RSD (%)	Accuracy (%)	n
80	79.1	7.3	−1.2	10
40	39.2	6.9	−2.0	10
5	4.8	6.1	−4.4	10
1	0.95	6.2	−4.5	10

Table 2
Inter-day precision and accuracy

Concentration level (ng/ml)	Measured concentration (average) (ng/ml)	RSD (%)	Accuracy (%)	n
80	81.0	11.9	1.3	6
20	19.9	12.8	−0.8	6
5	4.7	12.7	−5.6	6
1	0.99	9.6	−1.2	6

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