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# Identification of phase I and phase II metabolites of ketobemidone in patient urine using liquid chromatography–electrospray tandem mass spectrometry

Ingela Sundström[a], Ulf Bondesson[a,b], Mikael Hedeland[b,*]

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

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**Abstract**

Ketobemidone and five of its phase I metabolites were identified in the urine of four patients post intravenous administration of Ketogan Novum®. Furthermore, indications of the presence of the glucuronide conjugates of ketobemidone and norketobemidone is presented. Both hydrolyzed (β-glucuronidase) and unhydrolyzed human urine was extracted on a mixed-mode slightly polar cation-exchange SPEC cartridge prior to analysis with LC–ESI-MS–MS. The phase I metabolites were identified by comparison of their daughter spectra with those of synthesized standards. The glucuronides were identified by their molecular mass and interpretation of the daughter spectra, as no standards were available for these compounds.


*Keywords:* Ketobemidone

## 1. Introduction

Ketobemidone (structure see Fig. 1) is a narcotic analgesic, structurally related to pethidine (meperidine). It has found extensive use in Scandinavia and in some other European countries since the late 1940s for treatment of severe pain. The analgesic potency is claimed to be of the same order of magnitude as that of morphine [1]. Earlier studies have shown that ketobemidone has a similar effect and pharmacokinetic profile as morphine [2].

The amino phenolic narcotic analgesics morphine [3], pentazocine [4] and meptazinol [5] are mainly excreted in the urine of humans as metabolites. Morphine is mainly metabolized by *N*-demethylation to normorphine or conjugated to morphine-3-glucuronide or morphine-6-glucuronide [6]. These metabolites possess specific pharmacological activities and have been identified both in human and animal experiments. Another synthetic opioid used in treatment of moderate traumatic or postoperative pain is pethidine. The metabolic pattern of this compound has been reported in different studies identifying phase I metabolites and some conjugates [7]. One of the phase I metabolites is the hydroxymethoxy form, but the positions of the hydroxy and the methoxy functions have not been defined [8].

*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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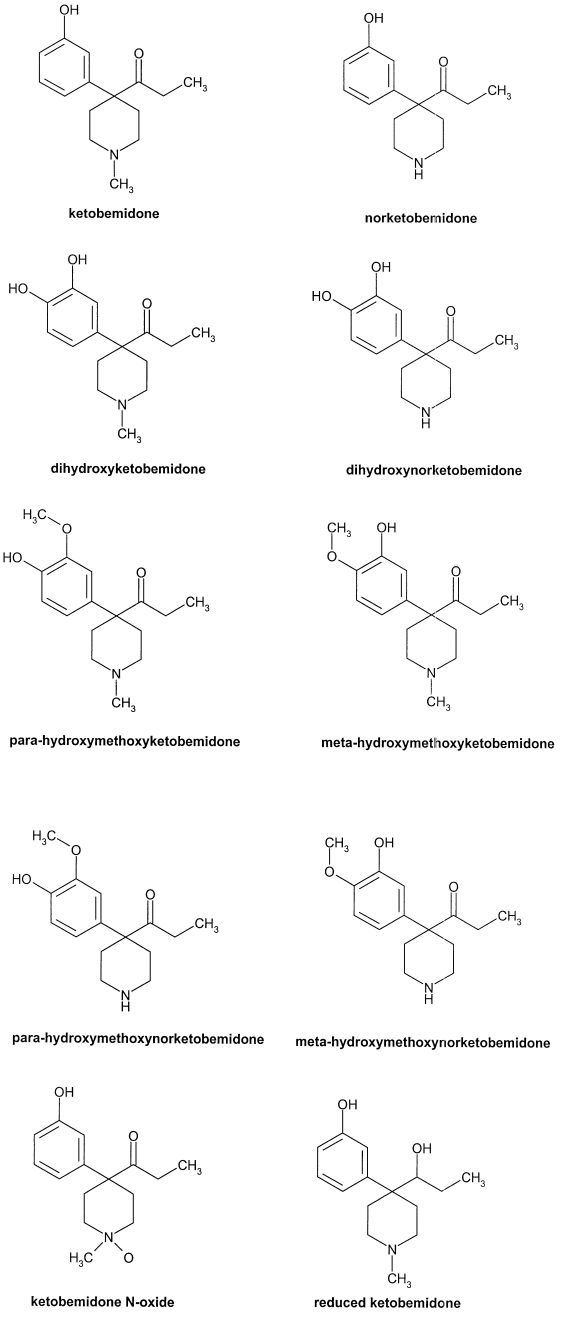


Fig. 1. Solute structures.

The metabolic pattern of ketobemidone has earlier been studied in healthy volunteers by GC–MS [9]. Three phase I metabolites; norketobemidone, dihydroxyketobemidone and *p*-hydroxymethoxyketobemidone were identified in this investigation. The simultaneous quantitative determination of ketobemidone and norketobemidone in patient plasma has also been accomplished using GC–MS [10]. Furthermore, a method for the quantitative analysis of ketobemidone and norketobemidone in the urine of drug abusers using LC–ESI-MS with a single quadrupole instrument, has been published [11]. Acid hydrolysis prior to analysis have given rise to an increased amount of detected ketobemidone and norketobemidone, indicating the presence of conjugated metabolites [9]. However, to the best of our knowledge, the nature of these conjugates has not yet been elucidated.

The aim of the present study was to identify phase I metabolites as well as glucuronide conjugates in patient urine by SPE followed by LC–ESI-MS–MS. In this way, the earlier problems with GC concerning thermolability and the need for derivatization could be avoided. Standards of ketobemidone and nine of its phase I metabolites were available for comparison of their daughter spectra to those of compounds found in human urine after intravenous ketobemidone administration.

## 2. Experimental

### *2.1. Chemicals*

Ketobemidone (1-[4-(3-hydroxyphenyl)-1-methyl-4- piperidyl]- 1- propanone) was obtained from Pharmacia (Uppsala, Sweden). The metabolites norketobemidone (1-[4-(3-hydroxyphenyl)-4-piperidyl]-1- propanone), dihydroxyketobemidone (1-[4-(3,4-dihydroxyphenyl)- 1- methyl- 4- piperidyl]- 1- propanone), *p*- hydroxymethoxyketobemidone ((1-[4-(4-hydroxy- 3- methoxyphenyl)- 1- methyl- 4- piperidyl]- 1- propanone), *m*- hydroxymethoxyketobemidone (1-[4- (3- hydroxy- 4-methoxyphenyl)- 1- methyl- 4- piperidyl]- 1- propanone) dihydroxynorketobemidone (1- [4- (3,4- dihydroxyphenyl)- 4- piperidyl]- 1- propanone) *p*- hydroxymethoxynorketobemidone (1- [4-(4- hydroxy- 3- methoxyphenyl)- 4- piperidyl]- 1- propanone), *m*- hydroxymethoxynorketobemidone (1- [4-(3- hydroxy- 4- methoxyphenyl)- 4- piperidyl]- 1- propanone) reduced ketobemidone (1- [4- (3- hydroxy-

phenyl)- 1- methyl- 4- piperidyl]- 1- propanol) and ketobemidone- *N*- oxide (1- [4- (3- hydroxyphenyl)- 1- methyl- 4- piperidyl]- 1- propanone- *N*- oxide were kind gifts from A/G Lundbeck (Copenhagen, Denmark). The structures are shown in Fig. 1.

The injection formula Ketogan Novum® (Pharmacia Sverige, Sweden) contains ketobemidone hydrochloride 5 mg/ml.

β-Glucuronidase *E. coli* strain K12 was purchased from Roche Diagnostics (Mannhein, Germany). The water was of Super-Q quality (Millipore, Bedford, MA, USA). All other reagents were of analytical or HPLC grade and used without further purification.

### 2.2. Administration to patients

Urine was collected from four patients treated with Ketogan Novum® used as pain control after coronary bypass surgery in a study containing 40 patients. The study was started after the approval by the hospital ethics committee and the informed patient consent. The administration of Ketogan Novum® was made intravenously from a patient-controlled analgesic pump. The sampling of urine started in the middle of the treatment and continued for some of the period.

### 2.3. Sample work-up

#### 2.3.1. Enzyme hydrolysis

A 1.0-ml volume of 1.0 *M* sodium acetate buffer (pH 4.8) and 100 μl β-glucuronidase solution were added to 2.0 ml of urine. The hydrolysis was performed at 50°C for 60 min. The pH was adjusted to 9.4 by addition of 1.0 ml of saturated carbonate buffer (pH 9.4) before decanting the sample onto the SPEC columns.

#### 2.3.2. Extraction

The solid-phase extraction columns, SPEC MP3 (mixed-mode slightly polar strong cation-exchange) microcolumn discs 15 mg (Division of Ansys, Irvine, CA, USA), were inserted into a vacuum manifold. The extraction columns were activated by addition of 200 μl of methanol. After 1 min, the hydrolyzed or unhydrolyzed urine samples were decanted onto the column and aspirated at ~1 ml/min. The columns were washed sequentially with 300 μl of water, 300 μl of 0.1 *M* acetic acid (aq) and 300 μl of methanol. The vacuum was increased and the extraction discs were dried for about 5 min. A 300-μl volume of a mixture of ethyl acetate and concentrated ammonia solution (98:2) was added and allowed to drip through the discs into the collection vials by applying a gentle vacuum. This procedure was repeated once. The eluates were evaporated to dryness under a gentle stream of nitrogen at 50°C. The residues were subsequently reconstituted in 80 μl of 0.1 *M* acetic acid in water.

### 2.4. LC–ESI-MS and MS–MS

An HP series 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany) with a binary pump, degasser and autosampler was used. The chromatographic column was a Luna $C_{18}$ (2) (Phenomenex, Torrance, CA, USA) with a particle diameter of 5 μm and dimensions of 150 mm×2.00 mm (length× I.D.), except in the last study, where a Synergi Polar-RP (Phenomenex, Torrance, CA, USA) with 4-μm particles and the same dimensions as above, was used. The mobile phase was 20% methanol in 0.1% acetic acid (aq.) in the experiments with the Luna column and 40% methanol in 0.1% acetic acid (aq.) in the experiments with the Synergi column. The volumetric flow-rate was 0.2 ml/min and the injection volume was 5.0 μl unless otherwise stated. The chromatography was performed at ambient temperature.

The column outlet was connected to a Quattro LC (Micromass, Manchester, UK) quadrupole–hexapole–quadrupole mass spectrometer equipped with an electrospray interface (ESI). The instruments were controlled by a PC using the software MASSLYNX v. 3.3. The MS parameters were optimized for sensitivity manually during direct infusion of a solution of ketobemidone standard. During the analyses, the ESI parameters were set as follows: capillary voltage 1.00 kV, cone 42 V, extractor 9 V and RF lens 0.80 V. The desolvation temperature was 350°C and the source block temperature 130°C. The desolvation and nebulizer gas flows were 575 l/h and 108 l/h, respectively. When running MS–MS, the hexapole collision cell was filled with argon at a

pressure of $7.6 \cdot 10^{-4}$ mbar. The collision energies were optimized in order to get selective daughter spectra for each compound where standards were available.

## 3. Results and discussion

### *3.1. LC–ESI-MS of synthesized standards of ketobemidone and its metabolites*

Documentation of chromatographic retention and daughter ion mass spectra of standards of ketobemidone and the possible phase I metabolites; norketobemidone, dihydroxyketobemidone, dihydroxynorketobemidone, *p*- and *m*- hydroxymethoxyketobemidone, *p*- and *m*- hydroxymethoxynorketobemidone, reduced ketobemidone and ketobemidone *N*- oxide was performed (see Fig. 1). The standards were spiked to blank urine at concentrations of 1–4 μ*M* and treated with enzyme hydrolysis and SPE as described in Section 2.3. All the added compounds were recovered and could be detected by LC–ESI-MS (Fig. 2). The identification was made by comparison with injected pure standards. As can be seen in this chromatogram, the compounds with equal molecular mass, e.g. the structural isomers *p*- and *m*- hydroxymethoxyketobemidone (*m*/*z* 278) and *p*- and *m*- hydroxymethoxynorketobemidone, dihydroxyketobemidone and ketobemidone *N*- oxide (*m*/*z* 264) were well separated. This was a prerequisite for their separate determination by MS.

Interestingly, the ketobemidone *N*-oxide gave rise to two peaks with slightly different daughter spectra (Figs. 2 and 5). It is well known that tertiary *N*-oxides are stereochemically stable and thus do not interconvert at room temperature [12]. The geometrical isomers of 1,4-diphenylpiperazine dioxide has been found to be stable and readily resolvable [12]. Furthermore, the nicotine metabolites *cis*- and *trans*-nicotine-*N*′-oxide have been possible to separate chromatographically [13]. Thus, it is possible that the two peaks observed in the present study were due to stable, separable *cis*-/*trans* isomers of ketobemidone *N*-oxide that were formed during the synthesis.

### *3.2. Phase I metabolites detected in patient urine*

Urine of four patients treated with ketobemidone was hydrolyzed with β-glucuronidase and extracted by the SPE method as described in Section 2.3. An example of an LC–ESI-MS chromatogram from the urine extract of one of the patients is shown in Fig. 3, where peaks at *m*/*z* values corresponding to

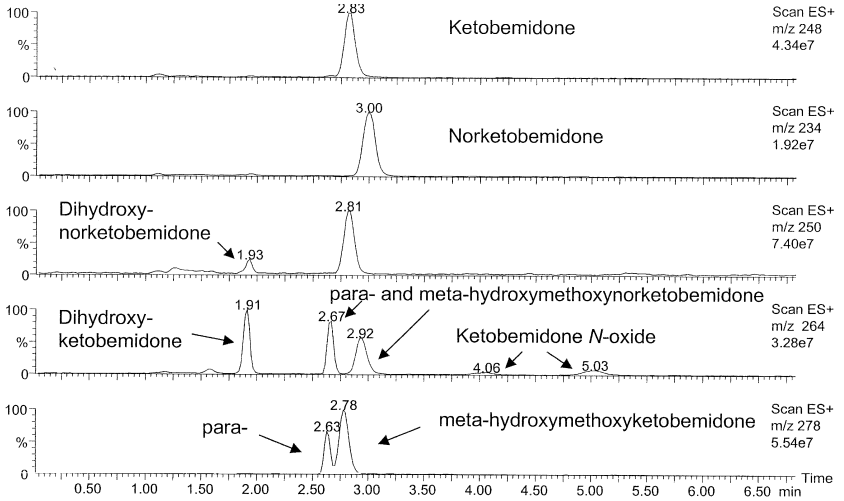


Fig. 2. LC–MS chromatograms of synthesized standards of ketobemidone and metabolites spiked to blank urine. Column: Luna $C_{18}$ (2). Conditions see Section 2.4.

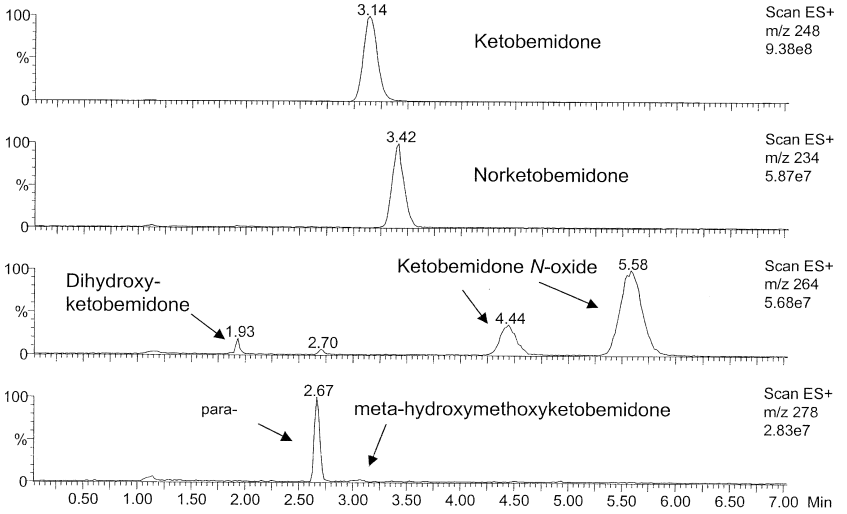


Fig. 3. LC–MS chromatograms of ketobemidone and phase I metabolites recovered from hydrolyzed patient urine after intravenous administration of ketobemidone (Ketogan Novum®). Column: Luna $C_{18}$ (2). Conditions see Section 2.4.

ketobemidone and some of its metabolites are visible. Subsequent LC–MS–MS analyses were performed in order to unambiguously identify the metabolites by comparing the daughter ion spectra of the peaks in the urine extracts with those of the synthesized standards. The presence of ketobemidone, norketobemidone and dihydroxyketobemidone in the patient urine could be confirmed in this way, Fig. 4.

Both peaks of the ketobemidone *N*-oxide observed in the synthetic standard compound also appeared in the chromatograms from the urine extracts, see Figs. 3 and 5. The daughter spectra of both peaks matched those of the standard well, Fig. 5. This indicates that also the metabolic *N*-oxidation of ketobemidone gives rise to a mixture of geometrical isomers. The presence of this metabolite could not be confirmed at all in the earlier study involving GC–MS [9].

A small peak eluting after the *p*-hydroxymethoxyketobemidone with a retention time close to the *m*-hydroxymethoxyketobemidone standard was observed in the urine extracts (Figs. 2 and 3), but the intensity of its daughter spectrum was too low for confirmation of the presence of this isomer. In order to increase the detectability, the injected sample volume was increased from 5 to 30 μl. This action was sufficient to obtain a clean spectrum which matched the standard well (Fig. 6). Thus, it was demonstrated that ketobemidone to some extent is metabolized to both structural isomers of the hydroxymethoxy form, however the para form being the dominant one. The metabolism of ketobemidone to *m*-hydroxymethoxyketobemidone was not reported in the earlier study [9]. Neither has the position of the methoxy group been defined for the corresponding metabolite of the structurally similar pethidine [8].

A peak at $m/z$ 264 in the urine extract with a retention time close to that of *p*-hydroxymethoxynorketobemidone standard ($t_R = 2.7$ min), was observed (Figs. 2 and 3). However, it was not possible to confirm the presence of this metabolite by MS–MS due to its low concentration. Furthermore, it was not possible to identify *m*-hydroxymethoxynorketobemidone or the reduced form of ketobemidone.

In summary, ketobemidone and the phase I metabolites norketobemidone, dihydroxyketobemidone, *p*- and *m*-hydroxymethoxyketobemidone and ketobemidone *N*-oxide were recovered in patient urine post hydrolysis. The two latter compounds have not earlier been reported [9]. These experiments demonstrate the power of the LC–MS–MS technique, as a chromatographic separation made it possible to identify metabolites with equal molecular mass (Figs. 2 and 3).

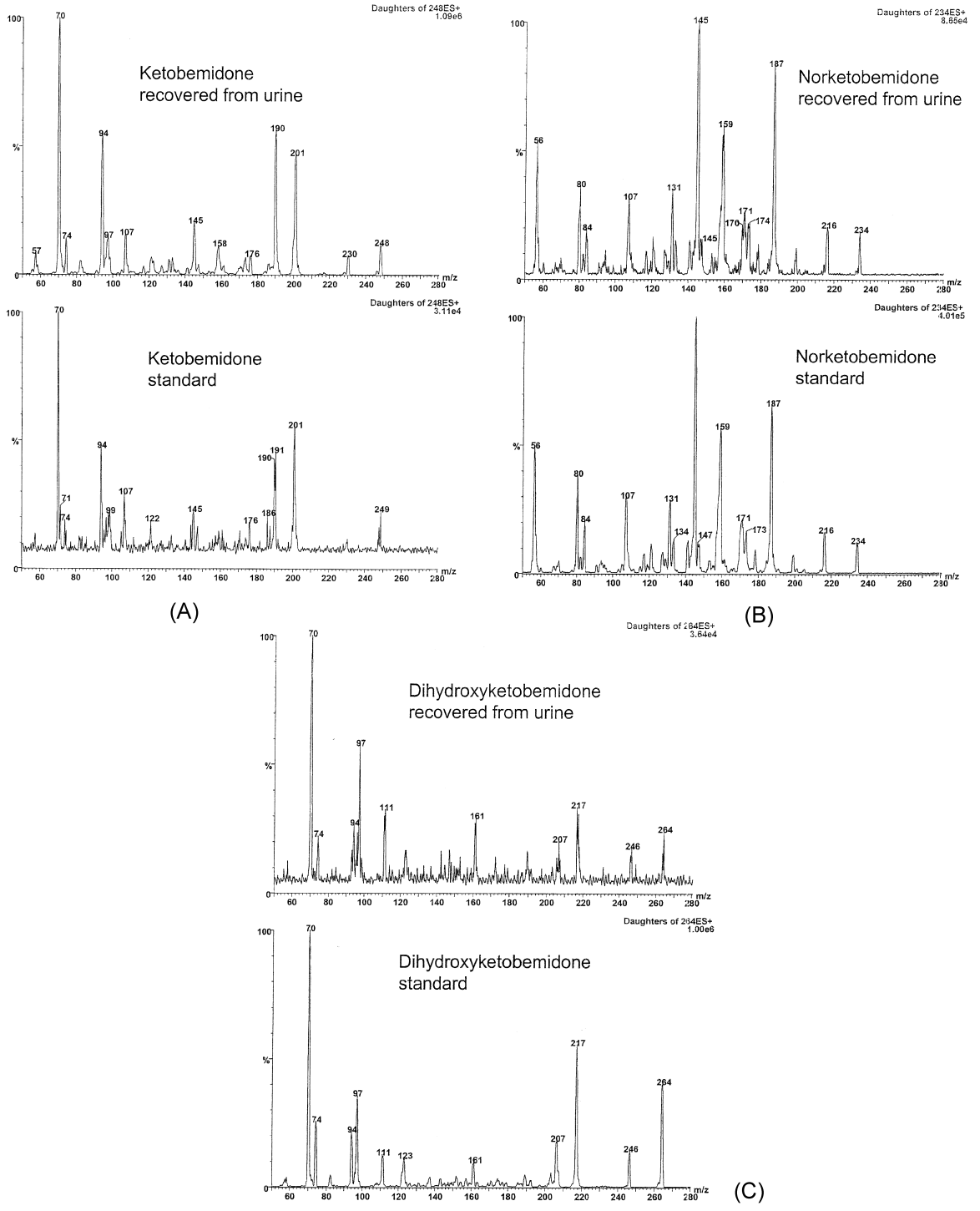


Fig. 4. LC–MS–MS spectra of substances recovered from hydrolyzed patient urine compared to synthesized standards: (A) ketobemidone, collision energy 28 eV, (B) norketobemidone collision energy 25 eV, (C) dihydroxyketobemidone collision energy 28 eV. Column: Luna $C_{18}$ (2). Conditions see Section 2.4.

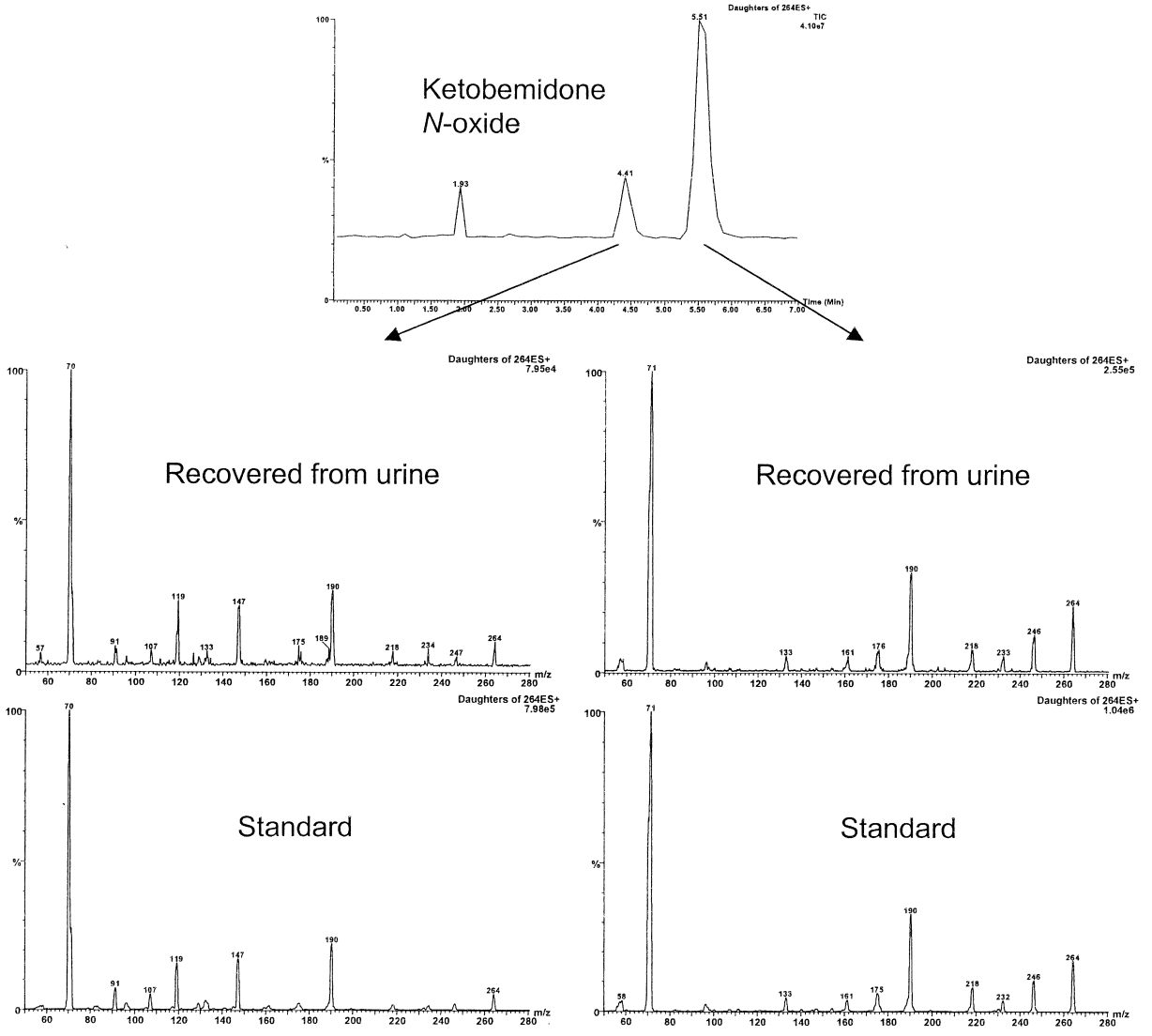


Fig. 5. LC–MS–MS chromatogram with the two peaks of ketobemidone *N*-oxide recovered from patient urine and LC–MS–MS spectra compared to synthesized standards. Collision energy 25 eV. Column: Luna $C_{18}$ (2). Conditions see Section 2.4.

### *3.3. Conjugated (phase II) metabolites of ketobemidone*

It has earlier been shown that the recovery of ketobemidone and norketobemidone from human urine increased markedly after acid hydrolysis [9]. This led to the conclusion that some kind of conjugates are formed in the body. The presence of glucuronic acid conjugates is well known from studies of the metabolism of other opioid analgesics such as morphine [6] and pentazocine [4]. However, the exact nature of the conjugates of ketobemidone has not yet been established. GC–MS was earlier the method of choice for metabolic studies of ketobemidone, which made it difficult to establish the presence of polar, nonvolatile conjugates.

In the present study with LC–ESI-MS, chromatographic peaks with $m/z$ values corresponding to the glucuronides of ketobemidone [$(M+H)^+$ $m/z$ 424] and norketobemidone [$(M+H)^+$ $m/z$ 410] were

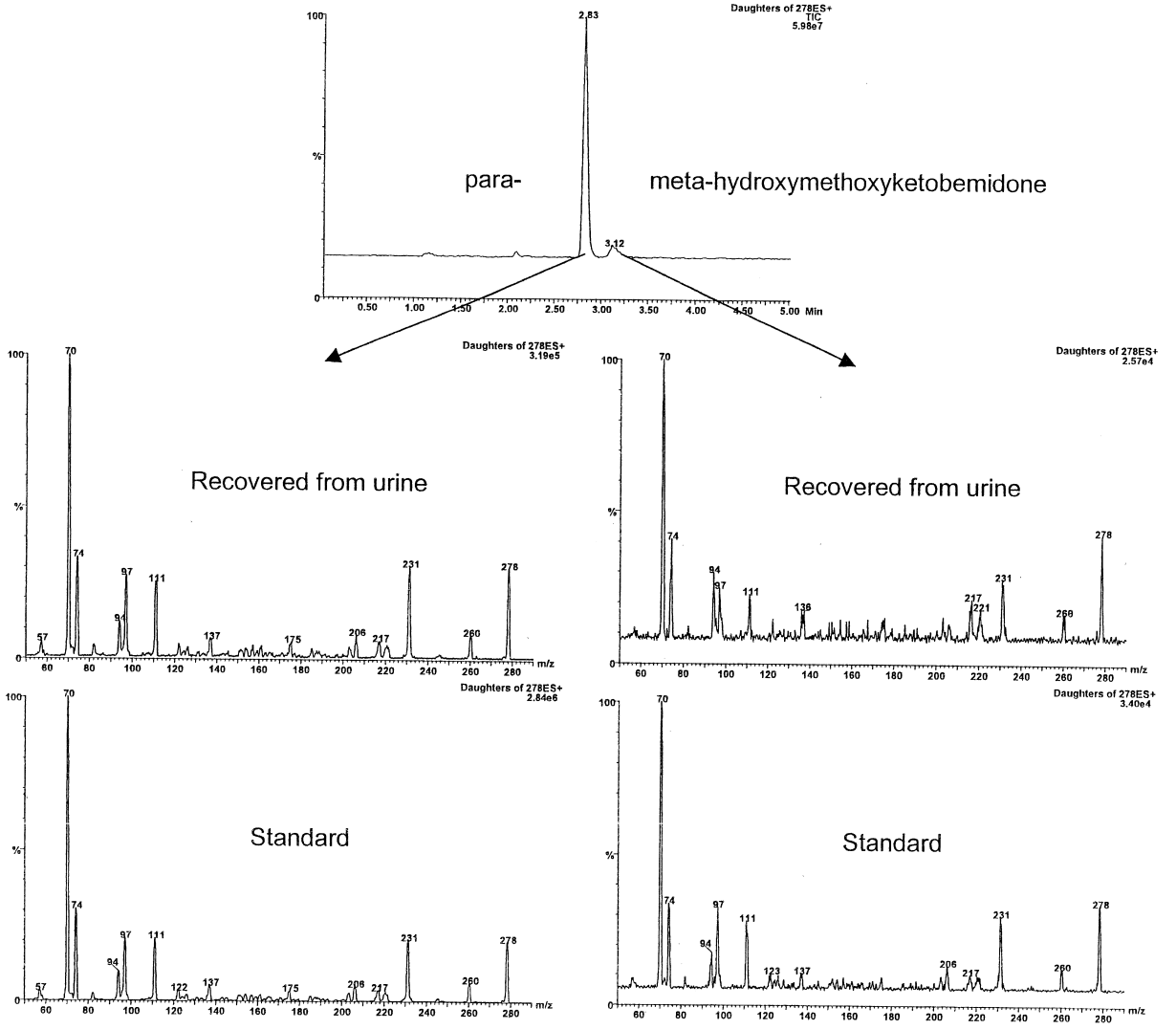


Fig. 6. LC–MSMS chromatogram of *p*- and *m*-hydroxymethoxyketobemidone recovered from patient urine and LC–MS–MS spectra compared to synthesized standards of *p*-hydroxymethoxyketobemidone and *m*-hydroxymethoxyketobemidone collision energy 25 eV. Column: Luna $C_{18}$ (2). Conditions see Section 2.4.

found in unhydrolyzed urine extracted as described in Section 2.3 (Fig. 7). The fragmentation of these compounds were investigated after argon collision. The daughter spectrum of the possible ketobemidone glucuronide had a main peak at $m/z$ 248, which corresponds to free ketobemidone and some lower mass fragments with $m/z$ equal to the fragments of the ketobemidone standard (Figs. 8A and 4A). The suspect norketobemidone glucuronide behaved correspondingly and gave a main fragment at $m/z$ 234 (equal to free norketobemidone) and some smaller fragments with $m/z$ identical to norketobemidone standard (Figs. 8B and 4B). These results indicate that these compounds are renally excreted as glucuronides after intravenous administration in humans. At the best of our knowledge, glucuronic acid

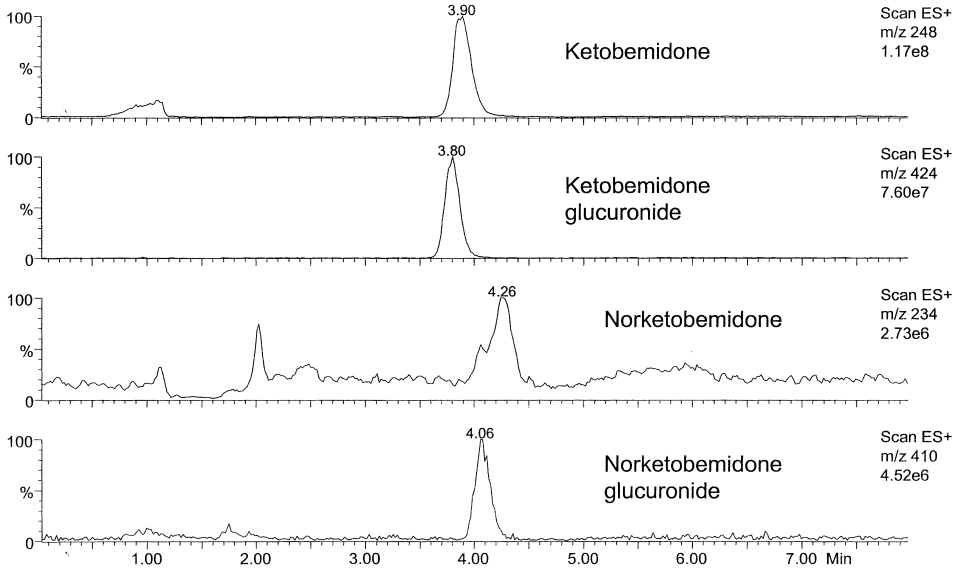


Fig. 7. LC–MS chromatograms of unhydrolyzed urine at the $m/z$ corresponding to ketobemidone, ketobemidone glucuronide, norketobemidone and norketobemidone glucuronide. Column: Luna $C_{18}$ (2). Conditions see Section 2.4.

conjugates of ketobemidone and norketobemidone have not earlier been identified. However, the presence of glucuronides of the other phase I metabolites or any other kinds of conjugates was not established in this study. Further optimization of the SPE method has to be performed in order to increase the recovery, as most of the glucuronides proved to be eluted in the washing step (results not shown).

Furthermore, there was an incomplete chromatographic resolution between the ketobemidone glucuronide and ketobemidone, as well as between norketobemidone glucuronide and norketobemidone on the Luna $C_{18}$ (2) stationary phase (Fig. 7). In order to enhance the chromatographic performance, another solid-phase called Synergi Polar-RP was tested on the unhydrolyzed urine extracts. This silica-based phase contains bonded ether-benzyl groups and is endcapped with a proprietary hydrophilic moiety. Both ketobemidone, norketobemidone and their respective glucuronides were substantially more retained on this polar phase compared to the $C_{18}$. Thus, a higher percentage of methanol in the mobile phase was needed to elute these compounds within a comparable time frame. Interestingly, there was a significantly better separation between the glucuronides and the parent compounds on Synergi Polar-RP compared to Luna $C_{18}$ (2) (Fig. 9).

## 4. Conclusions

The presence of the phase I metabolites norketobemidone, dihydoxyketobemidone, hydroxymethoxyketobemidone and ketobemidone *N*-oxide could be established in patient urine after intravenous administration of ketobemidone. The study was performed with a simple SPE method followed by LC–ESI-MS–MS. For the first time, it was shown that both structural isomers (*p* and *m*) of hydroxymethoxyketobemidone are formed in the human body. The ketobemidone *N*-oxide gave rise to two chromatographic peaks with slightly different daughter spectra possibly due to stable *cis*-/*trans* isomerism of this compound. This metabolite was not recovered in the former study [9]. Furthermore, there were indications of the presence of glucuronides of ketobemidone and norketobemidone. The nature of these conjugates has not been previously elucidated.

The method described in the present paper has several advantages compared to the previously de-

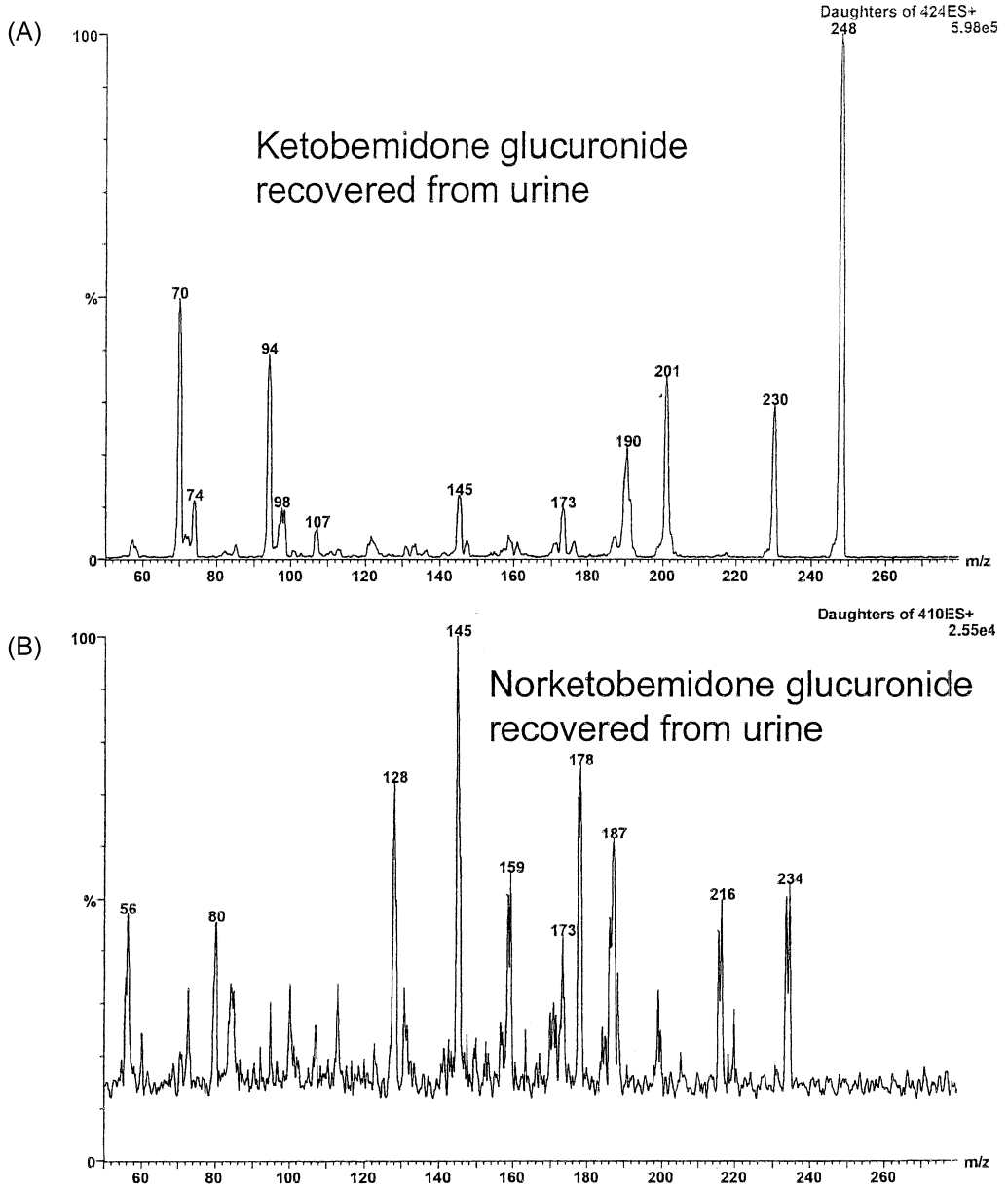


Fig. 8. Daughter spectra of the parent ions (A) $m/z$ 424 corresponding to ketobemidone glucuronide (collision energy 33 eV) and (B) $m/z$ 410 corresponding to norketobemidone glucuronide (collision energy 33 eV). Conditions see Section 2.4.

scribed GC–MS method, i.e. no demands on volatility, thermostability or derivatization of the samples. The present study also demonstrates the power of the combination of LC with MS, as a chromatographic separation made it possible to establish the presence of metabolites with identical molecular masses.

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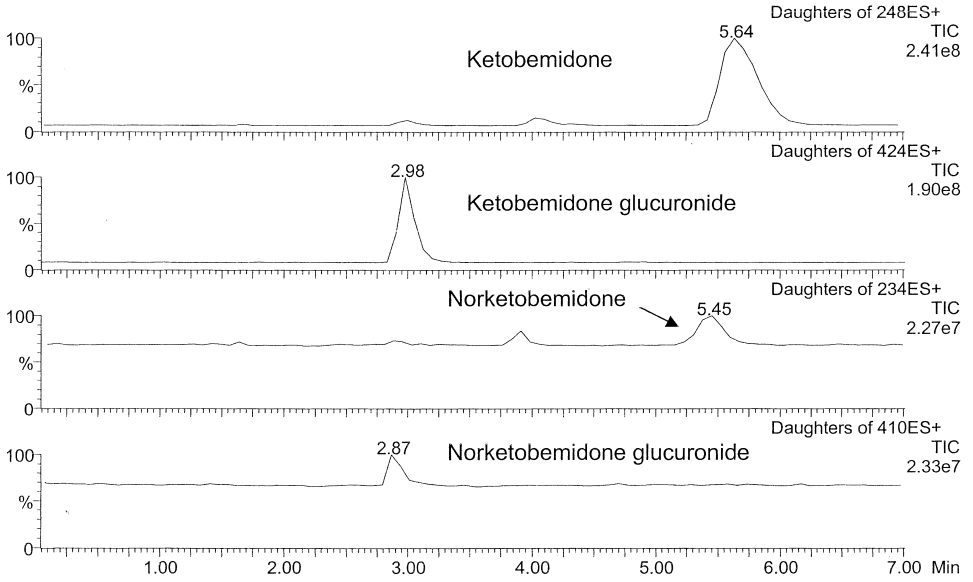


Fig. 9. LC–MS–MS chromatograms of unhydrolyzed urine at the $m/z$ corresponding to ketobemidone (collision energy 28 eV), ketobemidone glucuronide (collision energy 33 eV), norketobemidone (collision energy 25 eV) and norketobemidone glucuronide (collision energy 33 eV). Column: Synergi Polar RP. Conditions see Section 2.4.

Sweden) for lending us the Synergi Polar-RP column.

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