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Validation of urine drug-of-abuse testing methods for ketobemidone using thin-layer chromatography and liquid chromatography–electrospray mass spectrometry

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Abstract

High-performance thin-layer chromatography (TLC) with visual detection (post-chromatographic derivatization) was used in screening for the drug ketobemidone in human urine samples. High-performance liquid chromatography with electrospray mass spectrometry (LC–ESI–MS) was used for final confirmation of the result. The clean-up was performed by mixed-mode solid-phase extraction, and nalorphine was used as internal standard. A screening cut-off for TLC was established at 0.2 μg/ml. The mean recovery for LC–MS was 91% ($n=60$) with coefficients of variation (C.V.) in the range of 7 to 16%. Qualifying fragment ions of ketobemidone (m/z 190, 201 and 230) were generated by up front collision-induced dissociation (CID) on a single quadrupole instrument. Relative ion intensities were within ±15% deviation compared with standards in the same batch. The limit of detection for LC–MS was 0.025 μg/ml. Positive clinical samples from drug abusers ($n=10$) had concentrations in the range 0.07 to 3.2 μg/ml, which could be determined by LC–MS without matrix interference. During screening of unknown clinical samples ($n=27$) the results from TLC was in agreement with LC–MS data. After acid hydrolysis of conjugates in clinical samples the analyte response of ketobemidone and norketobemidone was increased by a factor of approximately two and twelve, respectively. A qualitative GC–MS technique was demonstrated for the detection of the spasmolyticum A29 (*N*,*N*-dimethyl-4,4-diphenyl-3-buten-2-amine), which can be found in a preparation combined with ketobemidone (Ketogan). © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Ketobemidone

1. Introduction

Ketobemidone is an opioid agonist and a potent narcotic analgesic, which has been used for decades in the treatment of severe pain (Fig. 1). It has also become a frequently abused drug in the Scandinavian countries, where it is registered under the trade names Ketogan, Ketodur and Ketorax. Ketogan also contain a spasmolyticum named A29 (*N*,*N*-dimethyl-4,4-diphenyl-3-buten-2-amine), which is not used in other drug formulations. Many cases of fatalities among drug addicts in Denmark have been related to overdoses of ketobemidone [1], which explains the fact that this drug is included in various urine drug-of-abuse screening programs. Since no immunoassay method is yet developed for ketobemidone and its metabolites, the initial screening has to be performed by alternative methods. As a consequence of this,

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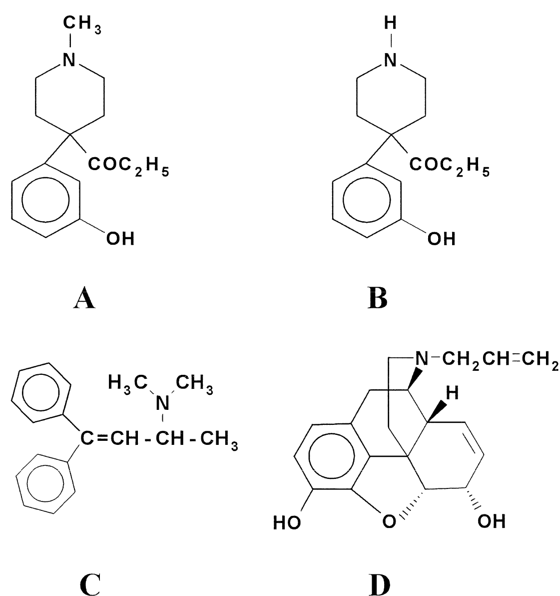

Fig. 1. Structures of (A) ketobemidone; (B) norketobemidone; (C) *N,N*-dimethyl-4,4-diphenyl-3-buten-2-amine (A29); (D) nalorphine (internal standard).

more than three thousand urine samples are analysed per year at our regional laboratory. Approximately 5% of these samples, mainly from drug addicts under treatment, are positive for ketobemidone.

Metabolic studies of ketobemidone and its major metabolites has been published by Bondesson et al. who developed the first quantitative research methods for plasma and urine using isotope dilution gas chromatography and mass spectrometry (GC–MS) [2–4]. These authors found that about 80% of a therapeutic oral dose (5 mg) was excreted into urine as ketobemidone and three principal metabolites, partly as conjugates. Unchanged ketobemidone constituted for about 44% of the total dose, and was eliminated rapidly, the major fraction being excreted during the first 6 h. About 40% of the fraction of ketobemidone was excreted in some conjugated form. The major metabolite, norketobemidone, constituted for about 19% of the mean urinary metabolite recovery.

Due to the high polarity of these compounds, it is essential to use derivatization prior to gas chromatography. As a first choice, methods based on liquid chromatography are therefore better suited for ketobemidone. In high-throughput screening, it is important to avoid slow column chromatographic procedures, where the time required for each analysis is additive. In this context specialized instrumentation like GC–MS or LC–MS should, preferably, be used for confirmative purposes. Otherwise unwanted bottleneck effects could be created for laboratories with big workloads, which would hinder the fast reporting times necessary for toxicology analysis.

The purpose of this study was to validate a fast TLC screening method and a safe confirmation method that was based on electrospray ionization mass spectrometry (ESI–MS), which could meet the general quality requirements and recommendations in the field of urine drug analysis including workplace drug testing [5,6]. A qualitative gas chromatographic–mass spectrometric method for the identification of A29, which is a marker for Ketogan administration, is also described.

2. Experimental

2.1. Chemicals

Ketobemidone, norketobemidone (desmethylketobemidone) and A29 (*N,N*-dimethyl-4,4-diphenyl-3-buten-2-amine) was a generous gift from Lundbeck (Valby, Denmark). Nalorphine (hydrochloride) was obtained from Sigma (St. Louis, MO, USA). A reference mixture of *n*-alkanes, C_{10}–C_{38} (100 μg/ml) was obtained from Promochem (Hertfordshire, UK). All other chemicals were of analytical reagent grade and used without further purification. Post-derivatization reagent for TLC was prepared fresh prior to use by slowly adding 5 ml of sodium nitrite (0.15 *M*) into 4 ml of nitroaniline (50 m*M*) and diluting to 100 ml with cold water (0–5°C). The nitroaniline solution (50 m*M*, pH≈1) was prepared by mixing 700 mg nitroaniline, 18 ml hydrochloric acid (6 *M*) and 20 ml water, heating to 60°C and finally diluting to 100 ml with water. Both stock solutions can be stored in the dark at 4°C for months.

2.2. Liquid chromatography–mass spectrometry

The liquid chromatograph was a Hewlett-Packard (Palo Alto, CA, USA) 1100 Series system with Chemstation software. The analytical column was a

reversed-phase Zorbax Eclipse XDB-C_8, 150×3.0 mm I.D, 5 μm, from Hewlett-Packard. Mobile phases were (A) 4 m*M* formic acid in water and (B) 4 m*M* formic acid in acetonitrile. The mobile phase conditions were as follows: 10% B for 1 min followed by a linear gradient to 80% B in 14 min. Then 80% B for 2 min and a linear gradient to 10% B in 2 min. Flow-rate was 0.5 ml/min. Column department temperature was 60°C. Injection volume was 0.2 μl. The quadrupole mass spectrometric detector was a Hewlett-Packard 1100 LC–MSD system equipped with an atmospheric pressure ionization electrospray interface. Selected ion monitoring (SIM) was performed in positive mode using the following combinations: [m/z/cone voltage (V)]: 248(110) $[\text{ketobemidone}+H]^+$, 234(80) $[\text{norketobemidone}+H]^+$, 312(80) $[\text{nalorphine}+H]^+$ and 190(150), 203(150), 230(150) [qualifying fragment ions of ketobemidone]. Dwell time was 114 ms. Capillary voltage was 4500 V. Drying gas was 99% pure nitrogen from a gas generator (Whatman, Haverhill, MA, USA) in line with 4000-150 BD air compressor from Junair (Nørresundby, Denmark). Drying gas temperature was 275°C and gas flow-rate 6.8 l/min. Nebulizer pressure was 248 kPa. Mass calibration (100–1000 amu) was performed using autotune macros and calibrators from the manufacturer. The calibration curve consisted of six calibration points at the concentrations 5, 10, 25, 50, 100 and 500 μg/ml. The internal standard concentration was 250 μg/ml. Calculations were based on peak area ratio of m/z 248 $[\text{ketobemidone}+H]^+$ relative to corresponding internal standard signal at m/z 312. Least-squares linear regression was used to fit the curves.

2.3. Thin-layer chromatography

Thin-layer chromatography was performed on 20×10-cm silica gel 60 F_{254} analytical HPTLC plates with 0.1-mm layers (Merck). The plates were activated at 110°C for 30 min, and kept until use in a desiccator with granulated silica gel. Standards and samples were applied 5 mm from the lower plate edge as 5-mm wide bands (volume 5 μl) by means of a TLC Sampler III (Camag, Muttenz, Switzerland). The total number of spots on a plate were eighteen. Plates were left to equilibrate for 10 min in a 20×10 cm horizontal TLC-chamber II (Camag). They were developed at a distance of 45 mm using methanol–ammonium hydroxide (25%) (100:1.5) as mobile phase. Developing time was approximately 9 min (22°C). Developed plates were left to air dry in a fume hood (5 min) and then dried on a TLC plate heater (Camag) at 70°C for 20 min. Post-chromatographic derivatization of ketobemidone was performed by fast exposure (1 s) of the plates in a dipping chamber filled with freshly prepared nitroaniline/nitrate reagent.

Densitometric scanning was performed at 283 nm using a TLC scanner II (Camag). Calculations of retention factors (R_f) were performed by the Camag CATS computer software (version 4.05).

2.4. Gas chromatography–mass spectrometry

Capillary gas chromatography was performed on a Varian (Palo Alto, CA, USA) Star 3400 gas chromatograph with a Varian Saturn II ion trap detector. The column was a Restek XTI-5 (30 m× 0.25 mm I.D., 0.25-μm film thickness) with a deactivated built-in guard column. Column oven temperature conditions were as follows: initial temperature 50°C was held for 1 min, then increased by 20°C/min to 170°C and finally increased by 9°C/min to 290°C, where it was held constant for 8 min. Transfer line was set at 290°C and ion trap manifold temperature was 250°C. Samples were injected in splitless mode (1 min) by a Varian 8100 autosampler into a 1077 injection port operated at 250°C. A double tapered insert liner (Restek) was used. Carrier gas was pure helium at 117 kPa. Mass spectra were recorded at m/z 50–500 (scan time 0.8 s). Retention index of A29 was calculated relative to n-alkanes, as $RI = 100n + 100(t_x - t_n)/(t_{(n+1)} - t_n)$, where n is the number of carbons in the n-alkane eluting before the analyte, t_x is the retention time of the analyte, t_n is the retention time of the alkane with n carbons and $t_{(n+1)}$ is the retention time of the alkane with $n+1$ carbons.

2.5. Sample preparation

Urine, 2 ml, was mixed with 1 ml ammonium acetate buffer (1 *M*, pH 8.8) and 50 μl of internal standard (nalorphine, 250 μg/ml) and centrifuged at

about 1000 *g* for 2 min. A vacuum manifold was used for solid-phase extraction. SPEC-PLUS-3 ML-MP1 (15 mg) columns (Ansys, Irvine, CA, USA) were conditioned with 200 μl methanol for 2 min (without vacuum applied). The sample supernate was transferred to and percolated through the column using a low vacuum (84 kPa). The column was washed sequentially with 500 μl water, 500 μl 0.1 *M* acetic acid and 500 μl methanol. Drying of the columns was performed in the manifold for a minimum of 5 min at 33 kPa. Finally the analytes were eluted with 900 μl of ethyl acetate–methanol–ammonium hydroxide (25%) (80:20:2). The eluate was evaporated to dryness in a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA) at a water bath temperature of 45°C using laboratory air at 103 kPa for 5 min. Analytes were redissolved in 50 μl acetonitrile, vortex shaken (5 s) and transferred to autosampler micro-vials.

Experiments with initial acid hydrolysis was performed by mixing samples with hydrochloric acid (10 *M*) in the ratio 6:1 and heating at 100°C in a pressure cooking device for 15 min. After hydrolysis, the sample pH was neutralized with 10 *M* NaOH and ammonium acetate buffer (1 *M*, pH 8.8) was added according to the standard method above.

2.6. Validation

The LC–MS method was validated for precision and accuracy by analysis of human urine samples (donor pool) spiked at six levels (0.2, 0.25, 0.5, 1, 2 and 4 μg/ml) in replicates of ten samples. The urine samples were spiked with ketobemidone and norketobemidone from a stock solution (5 mg/ml in acetonitrile) and stored in glass bottles at 4°C. The limit of detection (LOD) for LC–MS analysis was determined at a signal-to-noise-ratio (S/N) equal to 3 for qualifying ions.

A TLC scanning densitometer was used for the precise determination of R_f in the validation samples.

Positive clinical samples ($n=7$), that had been stored at −18°C, and routine clinical samples ($n=27$) were analysed by TLC and LC–MS. Internal quality control samples (QC, urine, 1 μg/ml) were prepared by the following procedure: Drug-free urine was centrifuged at 1000 *g* for 10 min, the supernate was separated and spiked with a stock solution of ketobemidone and norketobemidone.

Screening results for the major drug-of-abuse classes were obtained by enzyme multiplied immunoassay technique EMIT d.a.u. (Dade Behring, Deerfield, IL, USA).

3. Results and discussion

3.1. Solid-phase extraction

A micro solid-phase extraction (SPE) disc with a mixed sorbent phase (nonpolar/strong cation) was used to provide a very clean extract. The procedure, which can be regarded as a generic SPE method for basic drugs, was originally developed by the column manufacturer for extraction of morphine and codeine from hydrolysed urine [7]. During the second washing step equilibrium was shifted from alkaline to acidic conditions, thus making a final rinsing step with pure methanol possible. Thereby a coloured fraction of sample matrix was flushed out, while the target drug was retained by the cation ion-exchanger.

The compound nalorphine was chosen as internal standard. As a tertiary amine and phenol, it has some chemical similarity to ketobemidone. It is also very unlikely to find traces of this opioid antagonist in clinical samples. The deuterated internal standards of ketobemidone and norketobemidone that were synthesized by Bondesson et al. [8] are not commercially available.

3.2. Thin-layer chromatography

After a testing period, the mobile system methanol–ammonium hydroxide (100:1.5) was chosen because it showed good reproducibility. Systematic data compilations of retention factors (R_f) exist for this reference system [9], and it was also included in a comprehensive TLC and GC–MS drug screening program published by Lillsunde and Korte [10]. Preliminary results using ultra-violet densitometric detection (at 283 nm) failed due to major matrix interferences from coeluting drugs and metabolites in patient samples. However, the variation of R_f values for the validation samples in this study was precisely measured using this instrumental approach.

The mean R_f for ketobemidone for the calibration standard tracks ($n=60$) was 0.47 which is the exact reference value found in the literature [9]. The mean R_f for ketobemidone in validation samples was 0.44 ($n=60$). Norketobemidone and nalorphine had standard R_f values of 0.18 and 0.62, respectively. Variations of R_f for standards and validation samples were comparable and typically between one and two percent. Reporting of all screening results were based on a R_f window size of 5 mm, equivalent to ± 0.05 R_f. The LOD for ketobemidone using post-chromatographic derivatization was estimated to be 0.2 μg/ml. Blind samples ($n=10$) were free from analyte.

Clinical samples from drug addicts ($n=7$), previously verified positive for ketobemidone, and randomly selected routine samples ($n=27$) were analysed with the objective of testing for interference with endogenous compounds and drug metabolites. Immunoassay screening results (EMIT) performed on the clinical samples revealed contents of methadone metabolites (81%), benzodiazepines (56%), amphetamines (22%), opiates (22%), cannabinoids (14%) and barbiturates (4%).

The post-derivatization technique with a diazo reagent showed very good selectivity for these samples. Positive ketobemidone samples ($n=7$) were all correctly identified and three new positive screenings results where correctly assigned compared with LC–MS as reference method. The presence of internal standard in the samples and of ketobemidone in the QC sample was used for approval of each analytical batch analysed by TLC.

The diazo coupling reaction is very sensitive to pH. At mildly acidic or neutral solution, aromatic amines are reactive, but phenols like ketobemidone must be coupled at slightly alkaline conditions when they are converted to the more reactive phenoxide ions. However, at moderate alkaline conditions, the diazonium ion is converted into the deactive diazohydroxide. The best colouring reaction was, somewhat to our surprise, seen at pH$\approx$1 for the diazo reagent and when the plates where left to air dry for about 10 min. Based on these experimental facts, we must conclude that the general theory on diazo coupling reactions in solution [11] cannot be directly transferred to reactions which takes place on silica gel TLC plates. Ketobemidone and norketobemidone give bright orange or orange/red coloured bands, and nalorphine a red/brownish band, which slowly decolorizes. Methadone and spasmolyticum A29 does not react, whereas the major methadone metabolite (EDDP) gives a bright reddish band at $R_f=0.13$. The reaction is catalysed by exposure of the plates to ammonia vapours.

3.3. Liquid chromatography–mass spectrometry

Although GC–MS is still regarded as the standard instrumentation for confirmation and identification of unknown drugs after positive screening, LC–MS is also gaining importance as an increasing number of biomedical applications are made available. A problem to be faced by LC–MS users is the choice between MS–MS and single MS configuration to provide an unambiguous result. Molecular ion information is generally not sufficient for the identification of a compound. The successful employment of up-front CID ('cone fragmentation') to provide fragment ion information in a study of a cannabis metabolite in urine by ESI mass spectrometry was reported earlier [12]. Although the relative intensities of fragment ions changed between batches, they were within $\pm$20% derivation compared with standards in the same run. This tolerance is in accordance with the generally accepted guidelines for trace analysis by soft ionization techniques e.g. chemical ionization (CI). Preliminary studies obtained in our laboratory using a single quadrupole LC–MS system have shown that the intensities of CID ions for many drugs are only 5–30% relative to the parent ions. This may have a relative large effect on the LOD using qualifying ions, which could lead to an overall lower performance of LC–MS compared with GC–MS.

In the present study, a mobile phase with a low concentration of formic acid as modifier, made sensitive detection of protonated species of analyte and internal standard possible in ESI mode (Fig. 2). Three qualifying fragment ions of ketobemidone ($Q_1=m/z$ 230, $Q_2=m/z$ 201 and $Q_3=m/z$ 190) were generated by up-front CID using a selected-ion monitoring (SIM) program with individual cone voltages for each ion monitored. The fragment ions were 8–11% of the parent ion response (m/z 248). The relative intensities of qualifying ions for the

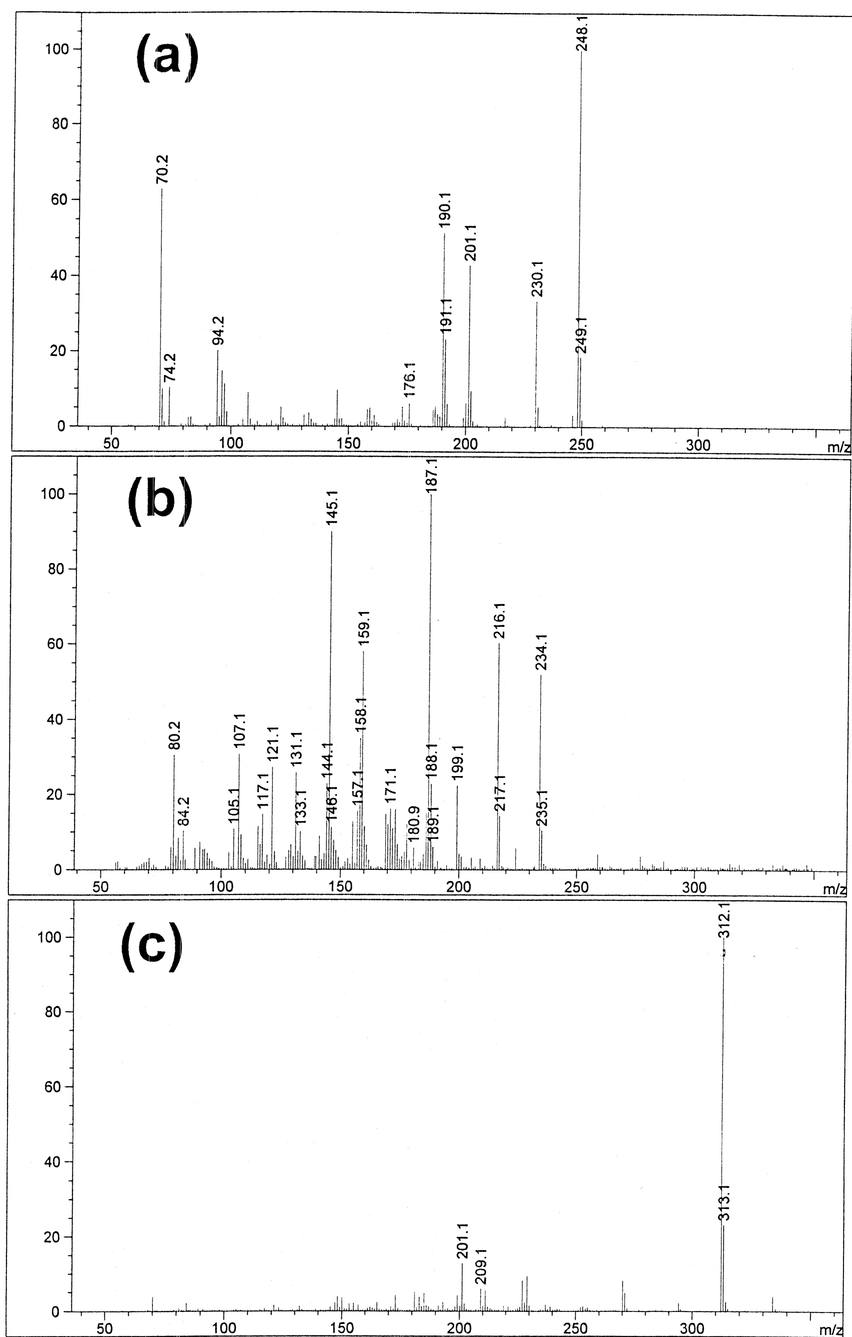

Fig. 2. Electrospray mass spectra of (a) ketobemidone; (b) norketobemidone; (c) nalorphine (internal standard). Data were obtained in full scan mode (m/z 50–500). Cone voltage: 150 V.

Table 1
Relative intensities (%) of qualifying ions (Q_1, Q_2 and Q_3) for ketobemidone in LC–MS analysis of spiked urine samples[a]

Concentration (μg/ml)	n	$Q_1 = m/z$ 230		$Q_2 = m/z$ 201		$Q_3 = m/z$ 190	
		Standards[b]	Samples[c]	Standards	Samples	Standards	Samples
0.20	10	7.7	8.1 (7.7–8.5)	9.1	8.8 (8.6–9.4)	11.1	10.77 (10.4–11.3)
0.25	10	7.9	8.3 (7.8–8.7)	8.6	8.2 (7.9–8.4)	10.5	9.8 (9.5–10.1)
0.50	10	7.9	8.2 (7.9–8.6)	8.6	8.6 (8.3–8.8)	10.5	10.6 (10.5–10.8)
1.0	10	8.1	8.6 (8.4–8.9)	8.7	9.1 (8.8–9.3)	10.5	10.8 (10.6–11.1)
2.0	10	8.1	8.3 (7.9–8.5)	8.7	8.9 (8.4–9.2)	10.6	10.9 (10.4–11.3)
4.0	10	8.1	8.7 (8.6–8.8)	8.7	9.5 (9.3–9.7)	10.6	11.6 (11.3–11.8)

[a] Qualifying ion intensities are calculated in percentage relative to m/z 248 $[\text{ketobemidone}+\text{H}]^+$.
[b] Mean values for the relative intensity of qualifying ions in calibration standards.
[c] Mean values for the relative intensity of qualifying ions in samples. High and low values are shown in brackets.

validation series were within ±15% deviation compared to standards in the same batch (Table 1). Norketobemidone co-eluted with ketobemidone, but did not give rise to any interference on qualifying ions. However, the opposite was the case for ketobemidone which had minor fragment ions interfering with the key fragments of norketobemidone (not shown in Fig. 2). A low extraction efficiency of norketobemidone (<50%) and a high degree of conjugation in real samples were the major obstacles for not including this metabolite in the final method. Nevertheless, monitoring of the protonated molecular ion of norketobemidone (m/z 234) in patient samples can be of value for qualitative analysis. Typical ion chromatograms are shown in Figs. 3 and 4.

The precision and accuracy for LC–MS (Table 2) was acceptable with coefficients of variation (C.V.) from 7 to 16%. Standard curves demonstrated correlation coefficients (r^2) of 0.999 or better. The equation obtained was typical: $y = 2.24x + 0.14$, where y is the area ratio and x the amount ratio of analyte to internal standard respectively. The mean recovery was 91% ($n = 60$) and the LOD was 0.025 μg/ml (corresponding to approximately 0.2 ng injected amount). Blind samples analysed by LC–MS ($n = 10$) had analyte concentrations below LOD.

Because ketobemidone is excreted in urine partly as conjugates, of which the structure is still unknown, the present method is limited to determine the fraction of free drug. It must be realized though, that in urine drug-of-abuse testing, quantification analysis is not strictly necessary nor meaningful, due to the fact that urine drug levels rarely bear any relationship to the amount of drug administered nor the time of administration. To test how hydrolysis of conjugates would affect the measured ketobemidone levels, two clinical samples, with ketobemidone concentrations of 3.2 and 0.87 μg/ml, respectively, were analysed in double after acid hydrolysis. Analyte signals of ketobemidone were increased with a factor of 2.0 and 2.1, respectively, which agrees with the observations of Bondesson et al. [3]. No interference was observed in the ion chromatograms for analyte and qualifying ions. The response of m/z 234 for norketobemidone was increased by a factor of twelve for the high level sample, indicating a high degree of conjugation for this metabolite. For the low level sample norketobemidone was only detectable after hydrolysis.

3.4. Internal quality control samples

External QC samples are not available for ketobemidone in any biological matrix. The internal QC samples used during final testing showed good stability at room temperature, however, no systematic stability test were performed. The C.V. for

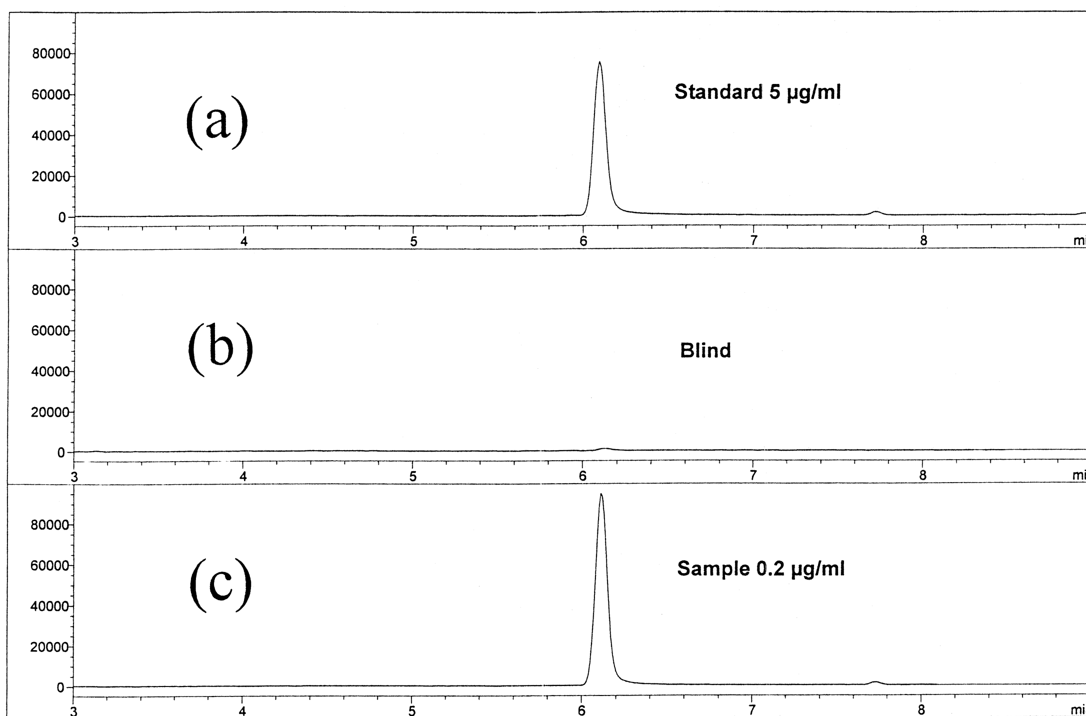

Fig. 3. Ion chromatograms of m/z 248 [ketobemidone+H]$^+$ in (a) calibration standard (5 µg/ml); (b) blind sample; (c) spiked urine sample (0.2 µg/ml).

QC samples measured by LC–MS in double in each batch was 5.7% ($n=10$).

3.5. Gas chromatography–mass spectrometry

Clinical samples screened positive for ketobemidone ($n=3$) were also tested positive for spasmolyticum A29 by GC–MS. Ketogan tablets contain 25 mg of A29 and 5 mg ketobemidone (as hydrochlorides). The plasma half lives of A29 are 3.0 and 3.8 h for intravenous and rectal administration, respectively, which was determined with GC–MS by Anderson et al. [13]. These values are slightly higher than for ketobemidone (plasma half lives 2.25, 2.5 and 3.25 h for intravenous, oral and rectal administration). Based on this similarity in pharmacokinetic profiles of the two drugs, and the five to one ratio in Ketogan formulations, we expect A29 to be detectable in the majority of positive patient samples after administration of Ketogan.

The compound is co-extracted in the present method, and can be detected by TLC at $R_f=0.52$. The response of the protonated molecular ion m/z 252 (R_t 9.3 min) in positive ESI spectra was very low due to extensive fragmentation. The same was true for the molecular ion signal in the ion trap mass spectrum (Fig. 5). The ion at m/z 236 can be explained by loss of CH_3 by alpha cleavage. The m/z 207 ion is present in ion trap mass spectra and to a low degree in electron impact (EI) spectra obtained by both GC–MS and direct inlet (probe) analysis. It is also observed as a base peak in ESI spectra (obtained at low cone voltage), where it can be explained by the elimination of $NH(CH_3)_2^+$ from $[M+H]^+$ to form a unique resonance stabilized carbocation. However, in the ion trap analyser where chemical ionization and CID reactions are possible, m/z 207 must be considered as an unusual artefact peak.

Representative chromatograms of A29 in a stan-

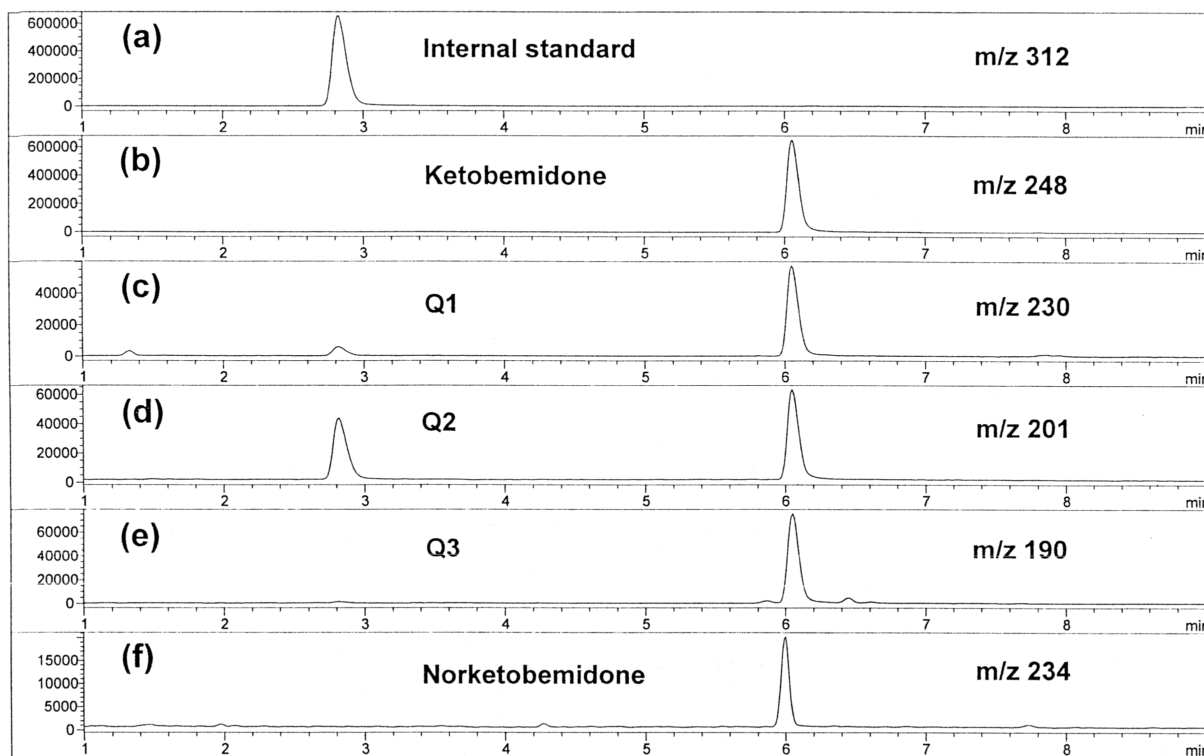

Fig. 4. Ion chromatograms from a clinical sample: (a) m/z 312 [nalorphine+H]$^+$ (R_t 2.82 min); (b) m/z 248 [ketobemidone+H]$^+$ (R_t 6.05 min); (c) m/z 230 [qualifying ion of ketobemidone=Q_1]; (d) m/z 201 [Q_2]; (e) m/z 190 [Q_3]; (f) m/z 234 [norketobemidone+H]$^+$ (R_t 6.00). The concentration of ketobemidone was 1.64 μg/ml.

dard and clinical sample is showed in Fig. 6. To the best of our knowledge, A29 is not included in any commercial reference mass spectra library. The retention index (RI) was determined to be 1918. In cases where A29 is found in urine samples, it is strongly indicative of ketobemidone administration and supports a positive confirmation of ketobemidone.

Table 2
LC–MS data for within-day precision (C.V.%) and efficiency of extraction from spiked urine samples

Concentration (μg/ml)	n	Mean recovery (%)	C.V. (%)
0.20	10	86	8
0.25	10	86	16
0.50	10	86	12
1.0	10	97	7
2.0	10	100	16
4.0	10	93	7

4. Conclusions

A TLC method using post-chromatographic derivatization reaction with a diazo coupling reagent was validated and found acceptable as an efficient screening method for ketobemidone in urine drug-of-abuse testing. Confirmation of the result by LC–MS on a single quadrupole instrument worked excellently using three qualifying fragment ions for identification. The spasmolyticum A29 can be used as a marker for Ketogan administration, and is easily detected by GC–MS. In the case of drug abuse, the estimated detection time for ketobemidone by TLC

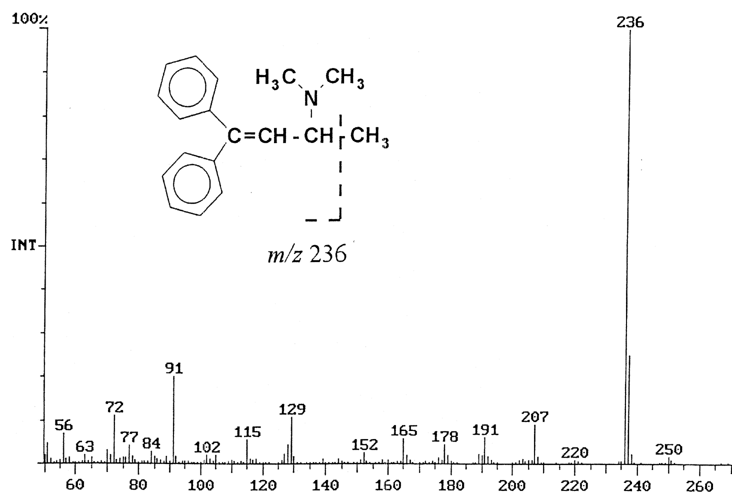

Fig. 5. Ion-trap mass spectrum of A29 (*N*,*N*-dimethyl-4,4-diphenyl-3-buten-2-amine).

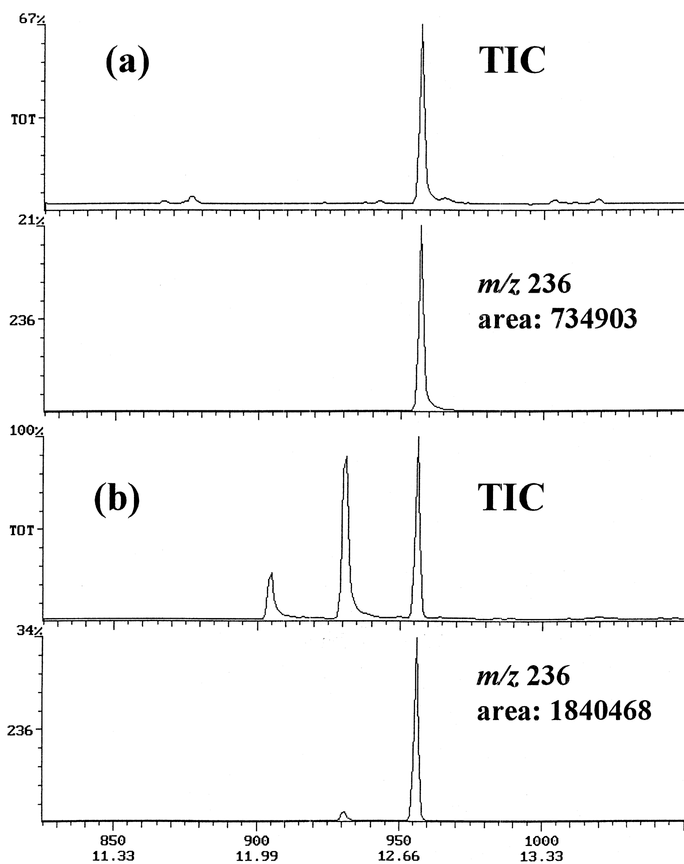

Fig. 6. Total ion chromatograms (TIC) and selected ion chromatograms (*m*/*z* 236) for GC–MS detection of A29 (R_t 12.75) in (a) calibration standard (3 μg/ml); (b) a clinical sample.

screening is 1–2 days after administration of the drug. However, it can be prolonged if an initial hydrolysis step is incorporated in the sample preparation.

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