Journal of Chromatography B, 877 (2009) 4055–4058

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Formation of the N-methylpyridinium ether derivative of propofol to improve sensitivity, specificity and reproducibility of its detection in blood by liquid chromatography–mass spectrometry

Detlef Thieme[a,*], Hans Sachs[b], Gustav Schelling[c], Cyrill Hornuss[c]

[a] Institute of Doping Analysis, Dresdner Str. 12, 01731 Kreischa, Germany
[b] Forensic Toxicological Center, Bayerstrasse 53, 80335 Munich, Germany
[c] Department of Anesthesiology, Klinikum Grosshadern, Ludwig-Maximilians-University, Marchioninistrasse 15, 81366 Munich, Germany

ARTICLE INFO

Article history:
Received 20 April 2009
Accepted 5 October 2009
Available online 12 October 2009

Keywords:
Propofol
Serum
Liquid chromatography–mass spectrometry
Derivatization
Pharmacokinetics
General anaesthesia

ABSTRACT

In spite of comparatively high therapeutic concentrations in blood, the quantitation of propofol may cause analytical challenges due to its pharmacokinetic (e.g. short half life, high distribution volume) and physicochemical (significant volatility) particularities. Moreover, a considerable and concentration dependent protein binding and a substance association to erythrocytes result in an irreproducible distribution between whole blood and serum. A possible redistribution in stored samples needs to be compensated during sample preparation or included into the result interpretation. The new analytical approach is based on a formation of N-methylpyridinium derivatives of propofol and corresponding internal standards and permits a significant (~300 fold) increase of detection limits in LC–MS/MS. Derivatization is achieved by a direct conversion of the acetonitrile supernatant of a protein precipitation with 2-fluoro-1-methylpyridinium-*p*-toluene-sulfonate using triethylamine as catalyst. The derivative exhibits a high solvent stability and provides – in contrast to the unchanged parent compound – a sufficient number of diagnostic qualifier fragments to fulfil common identification criteria. By using 2-tert-butyl-6-methylphenol as internal standard (instead of the commonly applied thymol), a better compensation of matrix effects could be achieved. Owing to its high robustness, appropriate quantitation limits (LLOQ ~ 13 ng/mL), minimum sample amount and preparation effort, the assay could efficiently be applied for quantitative propofol analyses in pharmacokinetic studies with high sampling rates.

1. Introduction

Propofol (2,6-diisopropylphenol) is a hypnotic agent widely used for intravenous induction and maintenance of general anaesthesia and for sedation during intensive care treatment.

Onset of propofol effects occur within seconds after injection and are terminated within minutes due to the high lipophilicity, large distribution volume of the substance and fast redistribution into peripheral tissues [1].

Examination of propofol plasma levels could be of particular interest for clinical as well as forensic reasons. Though the quantitation of propofol in blood is analytically favoured by its considerable high therapeutic serum concentrations far above detection limit, propofol quantitation bear some particular challenges both analytically and due to its pharmacokinetics.

Propofol is mostly bound to erythrocytes (50%) and serum proteins (48%, predominantly albumin) [2]. Therefore, therapeutic concentrations may vary inter-individually and are potentially influenced by individual constitution, i.e. body mass, gender, age, constitution (obesity) [3] or certain diseases (e.g. renal or hepatic disorders, hypoalbuminaemia). It is assumed that pharmacological effects are restricted to the availability of the unbound form. At low plasma concentrations, the proportion of free propofol tends to increase considerably [4].

Propofol concentrations obtained from conventional analytical procedures represent only the average amount in the respective fluid, e.g. serum, plasma total blood and may be affected by redistribution and deterioration during sample storage. Sample material needs to be highly standardised and uncontrolled sample deterioration (precipitation, haemolysis) has to be avoided. For quantitation of whole blood, an initial cell lysis by freezing [5] or solvent treatment [6] is suggested to efficiently account for the erythrocyte-bound proportion of propofol. Liquid–liquid (LLE [7,8]) or solid phase extraction (SPE [9,10]) and protein precipitation [5,11] represent the most frequent sample preparation protocols.

* Corresponding author. Tel.: +49 35206 2060; fax: +49 35206 20620.
E-mail address: detlef.thieme@idas-kreischa.de (D. Thieme).

1570-0232/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2009.10.010

Table 1
Ionization and fragmentation parameters of the relevant phenol–NMP derivatives.

Substance	Parent ion $(m/z)^+$	Declustering potential (V)	Product ion $(m/z)^+$	Collision energy (eV)
Propofol	270.1	51	133	35
			110	35
			91	43
2-Tert-butyl-6-methylphenol	256.1	51	105	39
			110	33
Thymol	242.1	51	105	39

The drawback of these extraction procedures consists in the necessity of evaporating organic solvents, which may be critical when dealing with volatile analytes. However, an alternative precipitation method was demonstrated to result in higher background levels than extractive pre-processing [12]. A crucial analytical problem of propofol detection in blood is due to its protein and erythrocyte binding and the resulting concentration discrimination between whole blood and serum. Quantitative uncertainties are mainly due to the high and inconsistent distribution rate (whole blood/plasma ratio = 1.64–2.02 [5]) and need to be taken into consideration, especially for kinetic studies.

In particular the concentration variation between whole blood, serum and sedimented cell fractions (cruor) needs to be considered and should be compensated by application of suitable internal references. Thymol appears to be the most widely used standard [5,7,8,10,13,14].

Various techniques for detection and quantification of propofol in whole blood and blood plasma have been successfully described using different approaches, e.g. gas chromatography–mass spectrometry (GC–MS) of the parent compound in whole blood [14] or after trimethylsilylation in plasma extracts [8], liquid chromatography (LC) [15] or fluorometric detection after deproteination [5] or LLE pre-processing [7]. The quantitation of propofol in alveolar gas appears to be a suitable clinical alternative due to its high correlation to whole blood concentrations [16,17].

The propofol plasma profile, however, is rapidly changing during induction of anaesthesia with reported changes in plasma concentrations between 2 and 10 μg/mL within less than 60 s [18]. Sole estimation of propofol plasma levels for pharmacodynamic studies through computer-based pharmacokinetic models could lead to flawed conclusions [18,19].

Analysis of propofol plasma levels for pharmacokinetic studies during induction and recovery from anaesthesia therefore require a frequent series of blood samples. For practical reasons, the amount of blood taken for propofol analysis should be as small as possible, thereby allowing quick sampling rates (e.g. collection every 5 s) in clinical studies.

Derivatization reactions prior to LC–MS/MS were reported to resolve similar analytical problems and provide sensitive and specific analytical procedures.

Functionalization of hydroxy groups (e.g. esterification of alcohol ethoxylates by phthalic anhydride [20]), amines and carboxy compounds using tris(trimethoxyphenyl)phosphonium compounds [21,22], conversion of phenolic hydroxy groups (e.g. estrogens by 2-fluoro-1-methylpyridinium salts, NMP [23,24]) or modification of oxosteroids by 2-hydrazino-1-methylpyridin [25] were reported to improve sensitivity of respective LC–MS/MS detection significantly.

In the present paper, the formation of a stable N-methylpyridinium ether derivative of propofol proved to increase the sensitivity and specificity of its detection and provides sufficient diagnostic fragments for an unequivocal identification. The gained sensitivity is ca. 300 times superior to unchanged propofol, analyzed under comparable conditions. This significant improvement would provide a considerable reduction of detection limits which is not clinically relevant. Alternatively, the amount of specimen could significantly be reduced, pre-processing was simplified to precipitation and sample dilution (instead of extraction) and chromatographic separation may be accelerated. These features permit an efficient application of the proposed assay in high throughput (e.g. pharmacokinetic) studies.

2. Experimental

2.1. Sample preparation

The internal standard 2-tert-butyl-6-methylphenol (tBuMPh, 99%, Sigma–Aldrich, Germany) is added to 50 μL of sample (whole blood, serum or cruor) to a final concentration of 1000 ng/mL. Alternatively, the suitability of thymol (>99.5%, Sigma–Aldrich) was tested. Propofol (2,6-diisopropylphenol > 97%) was purchased from SAFC (Germany).

Protein precipitation is achieved by addition of 1 mL acetonitrile (gradient grade, Baker, Germany) to 50 μL sample, subsequent vortexing and centrifugation. Reagents applied for derivatization were 2-fluoro-1-methyl-pyridinium-*p*-toluene-sulfonate (FluMP, purum, Aldrich, Germany) and triethylamine (purum, Riedel-de Haën, Germany). A working solution of 25 mg/mL FluMP in acetonitrile was freshly prepared prior to derivatization. To 20 μL of the supernatant, 40 μL of the FluMP solution and 10 μL of triethylamine were added. A color change to canary yellow is a reliable indicator for reagent stability and successful derivatization. After 10 min relaxation at room temperature, the triethylamine was removed by application of a gentle nitrogen stream at ambient temperature. Evaporation to complete dryness leads to deterioration of the derivative and must be avoided. The residue is reconstituted with 100 μL aqueous buffer and applied to LC–MS/MS.

2.2. Liquid chromatography–electrospray ionization-mass spectrometry

All LC–MS/MS analyses were carried out using an 1100 LC system (binary pump and autosampler, Agilent, CA, USA) coupled to an API 4000 mass spectrometer (Applied Biosystems, CA, USA), equipped with a Turbo-Ion-Spray (ESI) source. The instrument software Analyst (ver. 1.4.2) was used for data processing. Optimum ionization and fragmentation conditions of propofol and its analogues are summarized in Table 1.

The LC system was equipped with an Agilent Zorbax XDB-C8 analytical column (3 mm × 150 mm, 5 μm particle size).

The mobile phase consisted of (A) 0.1% (v/v) formic acid (AppliChem, Germany) with 5 mM ammonium formate (diluted from a 10 M aqueous stock solution, Fluka, Germany) and (B) methanol (gradient grade) containing 5 mM ammonium formate buffer and 0.01% (v/v) formic acid. A gradient program starting at a composition of 25% B, ramped to 80% B from 1 to 4 min with an isocratic post-run period (4–8 min) provided sufficient separation at a flow rate of 850 μL/min. A source temperature of 550 °C and gas flow settings (nitrogen as sprayer and heater gas) of 50 psi were applied. The injection volume was 10 μL. The assay was

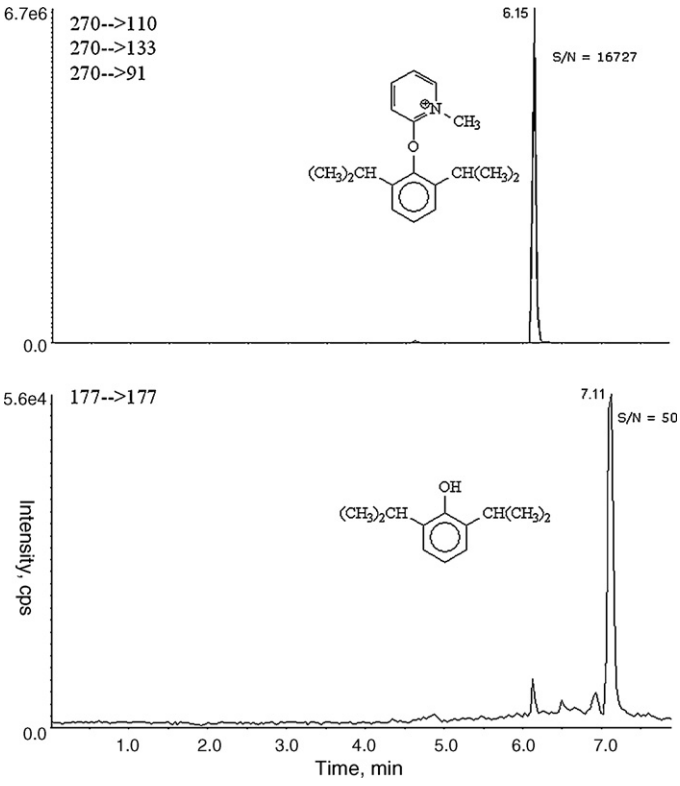

Fig. 1. Multiple reaction monitoring of equal concentrations of propofol with (top) and without (bottom) NMP-derivatization. The pseudo-MRM of propofol is characterized by poor specificity and lack of qualifier fragments. Formation of the N-methylpyridinium ether lead to ~300 fold increase of signal-to-noise ratios and similar improvement of detection limits and availability of sufficient qualifier fragments.

calibrated within a working range of 500–10,000 ng/mL. The intra-assay reproducibility was tested by duplicate injection of 5 sample aliquots, spiked to a target concentration of 1000 ng/mL.

3. Results and discussion

3.1. Formation of the N-methylpyridinium ether derivative

The drawback of upcoming LC–MS/MS assays for propofol quantitation consists in the weak fragmentation of the negatively charged $[M-H]^-$ pseudo-molecular ion ($m/z = 177$). Measures to overcome this limitation are monitoring of the intense but inspecific pseudo transition ($177 \rightarrow 177$ [10], Fig. 1) or the application of APCI to compensate for the relative low fragment intensity of $177 \rightarrow 161$ fragmentation [9]. Alternatively, Beaudry et al. [11] have utilized a propofol derivatization after protein precipitation to identify the resulting propofol-dansylate providing sufficient and abundant product ions in positive electrospray ionization (ESI) mode.

The chemical modification of a target compound (derivatization) to improve its ionization and/or fragmentation efficacy appears to be very common in GC–MS in contrast to LC–MS applications. However, in particular the introduction of permanently charged or polar substituents may improve the ionization of unpolar compounds (e.g. steroids) in ESI or APCI ionization modes.

The signal-to-noise ratio of the propofol N-methylpyridinium ether derivative proved to be about 300 times superior to the underivatized propofol (Fig. 1). The formation of permanently charged moiety improves the robustness by a significant increase of ion abundance and preventing the evaporation of the slightly volatile propofol during sample preparation.

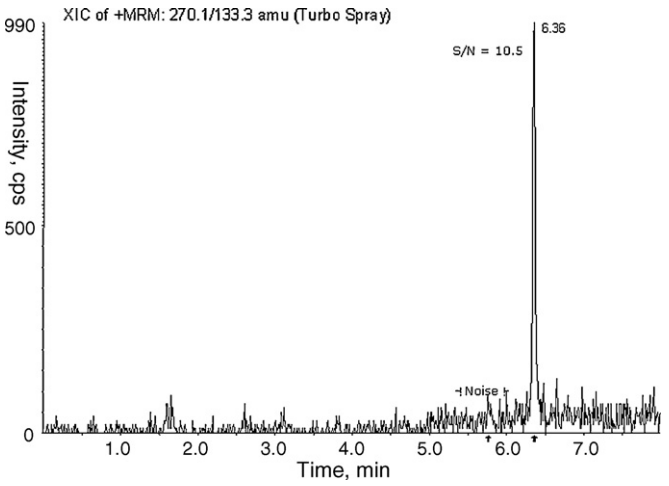

Fig. 2. Sub therapeutic trace amounts (e.g. 13 ng/mL) of propofol far below the working range, adjusted to clinically relevant concentrations between 500 and 10,000 ng/mL, may be detected.

Moreover, permanently charged analytes are generally less susceptible to ion suppression.

The working range of 500–10,000 ng/mL (e.g. whole blood, $r = 0.999$) reflects clinical requirements rather than technical limitations. During sample preparation, the original matrix is significantly (>100 fold) diluted with solvents corresponding with a low level of interference in the derivatized extracts. A propofol concentration of 13 ng/mL could be quantified at a signal-to-noise ratio better than 10 (Fig. 2). This value represents a reasonable estimation of a LLOQ, which is far below any clinical significance. The recovery of propofol from plasma samples was found to be 93.7%.

The intra-assay reproducibility of the concentrations in plasma samples, tested by 10 replicate measurements of a 1000 ng/mL standard was 6.0%. Due to irreproducible haemolysis and precipitation of blood cells, the homogeneity of whole blood is significantly deteriorated and the reproducibility of quantitative analyses is reduced to 15.6%.

3.2. Choice of internal standard

First approaches to quantify underivatized propofol in partially haemolytic blood samples were associated with limited reproducibility, most likely due to uncertain distribution of the analyte in the inhomogeneous matrix.

Therefore, the reproducibility of the assay and the suitability of internal standards to compensate for distribution effects were initially tested by analyzing a mixture of equal amounts of 1000 ng/mL propofol, thymol and tBuMPh spiked into a blood sample. The sample was repeatedly (5 aliquots each, double injections) analyzed in parallel as whole blood, serum and cruor samples. The relative standard deviation using tBuMPh as internal standard (15.6%) was gratifying, in contrast to unacceptable 75.9% for thymol. The origin of the higher discrimination between propofol and thymol may result from unequal distribution, extraction, absorption, or derivatization behavior and remained unclear. However, application of the modified technique, utilizing tBuMPh and subsequent formation of the N-methylpyridinium ether derivatives permits a sensitive, robust and reproducible detection of propofol in various sample materials.

3.3. Application to pharmacokinetic studies

The robustness of the essay was finally tested by its application to pharmacokinetic propofol studies [17]. The low blood sample

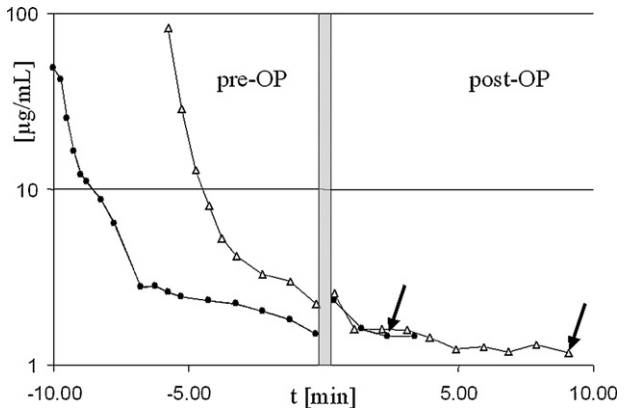

Fig. 3. Propofol concentration profiles in serum samples obtained from arterial blood of two patients. The negative time scale represents the propofol time course after an initial bolus injection. Positive time-values characterize its postoperative elimination. During surgery (variable time span, signified by grey bar), appropriate propofol levels were maintained by controlled flow injections. Time of awakening is signified by arrows. (Propofol concentrations greater than 10 μg/mL were confirmed by 1:5 dilution of the sample.)

demand, high reproducibility and robustness as well as comparatively low analytical effort permitted a recording of concentration profiles of propofol in arterial blood at high sampling rates (Fig. 3). Propofol concentrations measured after bolus injection or controlled infusion of propofol and subsequent elimination were in good accordance with clinical prospects. The initial distribution half life of propofol immediately after bolus injection (mean value of two patients) proved to be 1.1 min while an average of 11.9 min was observed during postoperative elimination.

These data are in good qualitative accordance to published pharmacokinetic studies [11,26] reporting a similar distribution and elimination kinetics. A characteristic distinction of this study was the occurrence of higher peak concentrations due to the application of arterial blood and short sampling intervals in the initial administration phase.

By formation of permanently charged N-methylpyridinium ether derivatives of propofol and its analogues, a swift, sensitive, specimen saving and robust quantitation method was established, which is particularly suitable for high throughput analyses (e.g. pharmacokinetic studies). The application of tBuMPh as internal standard is recommended to efficiently account for matrix effects in blood and related matrices.

References

[1] K. McKeage, C.M. Perry, CNS Drugs 17 (2003) 235.
[2] J.X. Mazoit, K. Samii, Br. J. Clin. Pharmacol. 47 (1999) 35.
[3] M. White, G.N. Kenny, S. Schraag, Clin. Pharmacokinet. 47 (2008) 119.
[4] A.L. Dawidowicz, R. Kalitynski, M. Kobielski, J. Pieniadz, Chem. Biol. Interact. 159 (2006) 149.
[5] X. Cussonneau, E. De Smet, K. Lantsoght, J.P. Salvi, M. Bolon-Larger, R. Boulieu, J. Pharm. Biomed. Anal. 44 (2007) 680.
[6] A.L. Dawidowicz, E. Fornal, Biomed. Chromatogr. 14 (2000) 493.
[7] K. Chan, A.P. So, Methods Find. Exp. Clin. Pharmacol. 12 (1990) 135.
[8] P.L. Stetson, E.F. Domino, J.R. Sneyd, J. Chromatogr. 620 (1993) 260.
[9] L. Bajpai, M. Varshney, C.N. Seubert, D.M. Dennis, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 810 (2004) 291.
[10] S. Cohen, F. Lhuillier, Y. Mouloua, B. Vignal, P. Favetta, J. Guitton, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 854 (2007) 165.
[11] F. Beaudry, S.A. Guenette, A. Winterborn, J.F. Marier, P. Vachon, J. Pharm. Biomed. Anal. 39 (2005) 411.
[12] A.L. Dawidowicz, A. Fijalkowska, J. Chromatogr. Sci. 33 (1995) 377.
[13] G.F. Plummer, J. Chromatogr. 421 (1987) 171.
[14] J. Guitton, M. Desage, A. Lepape, C.S. Degoute, M. Manchon, J.L. Brazier, J. Chromatogr. B: Biomed. Appl. 669 (1995) 358.
[15] I. Pavan, E. Buglione, M. Massiccio, C. Gregoretti, L. Burbi, M. Berardino, J. Chromatogr. Sci. 30 (1992) 164.
[16] M. Grossherr, A. Hengstenberg, T. Meier, L. Dibbelt, K. Gerlach, H. Gehring, Anesthesiology 104 (2006) 786.
[17] C. Hornuss, S. Praun, J. Villinger, A. Dornauer, P. Moehnle, M. Dolch, E. Weninger, A. Chouker, C. Feil, J. Briegel, M. Thiel, G. Schelling, Anesthesiology 106 (2007) 665.
[18] M.M. Struys, M.J. Coppens, N. De Neve, E.P. Mortier, A.G. Doufas, J.F. Van Bocxlaer, S.L. Shafer, Anesthesiology 107 (2007) 386.
[19] D.M. Fisher, Anesthesiology 107 (2007) 367.
[20] C.J. Sparham, I.D. Bromilow, J.R. Dean, J. Chromatogr. A 1062 (2005) 39.
[21] S.J. Barry, R.M. Carr, S.J. Lane, W.J. Leavens, S. Monte, I. Waterhouse, Rapid Commun. Mass Spectrom. 17 (2003) 603.
[22] W.J. Leavens, S.J. Lane, R.M. Carr, A.M. Lockie, I. Waterhouse, Rapid Commun. Mass Spectrom. 16 (2002) 433.
[23] T. Higashi, K. Shimada, Anal. Bioanal. Chem. 378 (2004) 875.
[24] J.M. Quirke, G.J. Van Berkel, J. Mass Spectrom. 36 (2001) 1294.
[25] T. Higashi, Y. Shibayama, K. Shimada, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 846 (2007) 195.
[26] S. Emara, G. Saleh, M. Fathy, M.A. Bakr, Biomed. Chromatogr. 13 (1999) 299.