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High-performance liquid chromatographic assay to detect hydroxylate and conjugate metabolites of propofol in human urine

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Abstract

This paper describes a HPLC method for the simultaneous detection of phase I (2,6-diisopropyl-1–4-quinol and 2,6-diisopropyl-1–4-quinone) and phase II (4-(2,6-diisopropyl-1–4-quinol)-sulphate, 1-(2,6-diisopropyl-1–4-quinol)-glucuronide, 4-(2,6-diisopropyl-1–4-quinol)-glucuronide, and propofol-glucuronide) metabolites of propofol in human urine samples. Separation was based on a simple mobile phase and a reversed-phase chromatographic column. Metabolite identification was performed by UV spectrum on a diode-array detector and by LC–APCI–MS. The identification was also carried out using in vitro incubation mixtures (cytosol and microsomes prepared from liver) from several species: human, rat and rabbit. This assay was performed using UV, fluorescence and electrochemical detection modes. Each of these was analyzed and discussed.

Keywords: Propofol; Metabolite; Glucuronidation; Sulfation

1. Introduction

Propofol is an intravenous anaesthetic used for the induction and maintenance of anaesthesia and sedation. The drug has a high total body clearance mainly by hepatic metabolism but also with extrahepatic sites of metabolism [1–4]. In humans, propofol metabolism consists of direct conjugation of the parental compound, leading to propofol-glucuronide (PG), or of hydroxylate metabolite (2,6 diisopropyl-1–4-quinol) leading to three metabolites, 1-quinol glucuronide (1-QG), 4-quinol glucuronide (4-QG) and 4-quinol sulphate (4-QS) (Fig. 1) [1,5–10]. About 90% of an injected dose of propofol is eliminated in urine in the form these four conjugated metabolites [5,7].

Methods for propofol assaying have been reported using high-performance liquid chromatography (HPLC) with UV [7,8,11–14], fluorescence [2,15–19], or electrochemical detection [20–23], or using gas chromatography (GC) coupled with a flame ionization detector [19,24], a mass spectrometer [25,26] or an atomic emission detector [27].

Assays for quantification of phase I metabolites

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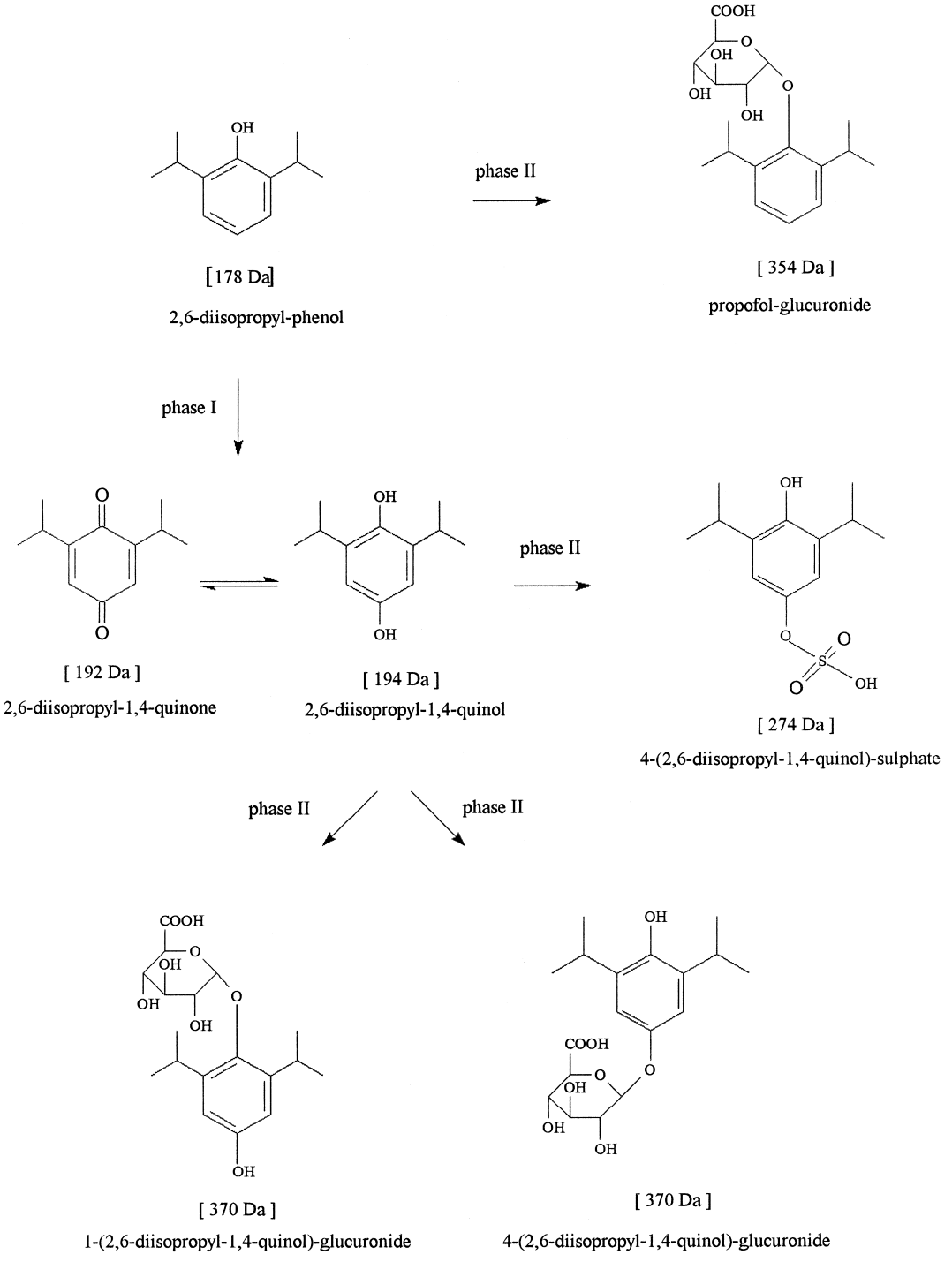

Fig. 1. Structures of propofol and its metabolites (described in human species) with their molecular weights.

have been also described using HPLC or GC techniques [14,25]. Characterization of the conjugates has been performed by Simons and co-workers [5,7,10]. These authors also described an assay based on proton NMR spectroscopy. An HPLC method to measure PG, synthesized from microsomal mixtures using [^{14}C]UDPGA, has also been proposed [1]. In both cases, anion-pairing chromatographic techniques have been used. More recently, an HPLC assay for the determination of 1-QG, 4-QG and PG in human plasma and urine has been reported [28].

The aim of this study is to develop a simple and current HPLC procedure in order to perform the simultaneous identification of human propofol metabolites (phases I and II). Moreover, we are comparing the classical HPLC detection methods (UV, fluorimetric or electrochemical detection) to measure the levels of propofol and its metabolites.

2. Experimental

2.1. Chemicals and reagents

Propofol (2,6-diisopropyl phenol) and its hydroxylate metabolite 2,6-diisopropyl-1–4-quinol (1–4 quinol) were kindly provided by Zeneca Pharma (Cergy, France). Thymol, phenyl-β-D-glucuronide, phenol, uridine diphosphoglucuronic acid (UDPGA), 3′-phosphoadenosine 5′-phosphosulphate (PAPS), dithiothreitol (DTT), β-glucuronidase/arylsulphatase (Helix Pomatia, Type H1), acetic acid, sodium phosphate dibasic heptahydrate and potassium phosphate monobasic were obtained from Sigma (St. Quentin Fallavier, France). HPLC-grade acetonitrile used in chromatography was purchased from Merck (Darmstadt, Germany).

2.2. Urine specimen

Urine samples were obtained from healthy human volunteers (blank urine) and from patients who received a propofol perfusion during ear surgery. Urine samples were collected over 6 h in a plastic bag via a catheter. Urine specimens were aliquoted and stored at −20°C prior to analysis.

Urine samples were centrifuged for 10 min at 3000 *g* or filtered on filter Millex-HA, 0.45 μm (Millipore, Bedford, USA) to remove particles in suspension. Several assays were carried out with pure urine. In this case, 10 μl were directly injected into the chromatographic apparatus. Extraction procedure was also carried out according to the following procedure: a 3-ml C_{18}, Sep-Pak, SPE cartridge (Waters, Milford, USA) was conditioned under vacuum with 2.5 ml of methanol and 2.5 ml of deionized water. Urine (5 ml) was poured into the cartridge. After a slow percolation, the cartridge was washed with 2.5 ml of deionized water. Elution was carried out with 0.5 ml of methanol. The methanolic eluate was divided into three equal aliquots. Each of them was evaporated to dryness at room temperature under a nitrogen gas stream. One aliquot was used as a control and the others underwent either enzymatic or chemical hydrolysis.

2.2.1. Control

The reconstitution was performed with 150 μl of mobile phase and 1 μl of the mixture was injected into the analytical column.

2.2.2. Enzymatic hydrolysis

The residue was reconstituted with 150 μl of acetate buffer (pH 5) containing 2.5 mg of β-glucuronidase/arylsulphatase (348 100 units/g solid). The solution was incubated at 37°C for 40 h. The tubes were placed in an ice-bath to stop the reaction. The mixture was then centrifuged at 4000 *g* for 15 min, and 1 μl of the clear supernatant was injected into the column.

2.2.3. Chemical hydrolysis

The residue was reconstituted with 150 μl of acetate buffer (pH 5), and pH was adjusted to 1.0 with 11 N HCl. The mixture was then transferred into a tightly sealed vial and placed in a thermostatic bath at 100°C. After 1 h, the urine was cooled in cold water and the pH adjusted to approximately 5.0 with NaOH 11 N. Centrifugation and injection conditions were the same as above.

2.3. In vitro studies

2.3.1. Glucuronidation

The study of glucuronide metabolites was carried out on rat, rabbit and human liver microsomes, all

prepared as previously described [25]. The reaction mixture (300 μl final volume) contained 100 m*M* Tris–HCl, pH 7.4, 10 m*M* $MgCl_2$, 1.5 mg microsomal proteins, 6.6 m*M* UDPGA and propofol or 1–4-quinol. For activation, microsomes were preincubated with Triton X-100 (10% in water, v/v) at 4°C for 1 h. The mixture was incubated at 37°C for 90 and 180 min. The reaction was stopped by adding 200 μl of methanol. The tubes were then centrifuged at 4000 *g* for 15 min. The supernatant was directly injected into the chromatographic column (5 μl per assay) or underwent a chemical or an enzymatic hydrolysis as previously described for urines.

2.3.2. Sulphation

Assay of sulphonation activity was carried out on rat liver cytosol. Incubation mixture contained 50 m*M* Tris–HCl, pH 7.4, 0.0625% bovine serum albumin (w/v), 8 m*M* DTT, 0.1 m*M* PAPS, 1 m*M* 1–4-quinol and cytosol [29]. Tubes were incubated for 15, 30 and 45 min at 37°C.

2.4. Instrumentation

2.4.1. UV detection

The HPLC system consisted of a HP 1100 series (Hewlett-Packard, Palo Alto, USA), equipped with a diode-array detector (DAD) model G1615A, a HP G1322A on-line degasser, a HP G1313A auto sampler and a HP G1311A pump. The chromatographic separations were carried out in linear elution gradient mode and on an Interchrom stainless steel column (250 mm length×4.6 mm I.D.) packed with Spherisorb 5 μm ODS 2 (Interchim, Montluçon, France). The solvents used were: solvent A, water, acetic acid, pH 3.8; solvent B, acetonitrile. Elution was performed with a linear gradient profile where solvent B varied from 13 to 89% in 35 min. The mobile phase was delivered through the column (temperature maintained at 25°C) at a flow-rate of 0.8 ml min^{-1} and the eluate was monitored using UV detection at 230 and 270 nm simultaneously.

2.4.2. Fluorescence detection

Chromatographic instrumentation and conditions were the same as described for UV detection except for the DAD which was replaced by a fluorescence spectrophotometer, model HP G1321A. The excitation and emission wavelengths were 276 and 310 nm, respectively.

2.4.3. Electrochemical detection

The HPLC system included a LC-10A pump (Shimadzu, Japan), an ESA Coulochem II (Bedford, USA) electrochemical detector coupled with dual electrode analytical cell (model 5011) and a guard cell (model 5020). The detector was connected to a C-R6A integrator (Shimadzu). Electrode potentials were set at +1 V for the guard cell and +400 and +650 mV (or +850 mV) for detectors 1 and 2, respectively. Samples were injected into a Rheodyne valve (model 7125) fitted with a 20-μl loop. The analytical column was the same as the one used for UV detection. Elution was isocratic at a flow-rate of 1 ml min^{-1}. The mobile phase consisted of acetonitrile–25 m*M* phosphate buffer, pH 3.8 (60:40, v/v), for standards (phenyl-β-D-glucuronide, phenol, propofol, 1–4-quinol) and (25:75, v/v) for urine extracts.

2.4.4. Mass spectrometry detection

Chromatographic conditions (analytical column, composition of mobile phase and elution mode) were the same as previously described for UV detection. A VG platform Biotech (Micromass, Manchester, UK) equipped with an APCI source was used. Source and probe temperatures were set at 150 and 400°C, respectively. Corona discharge voltage was maintained at 3 kV, and cone voltage was set at −30 or −60 V. The mass spectrometer scanned 100–450 atomic mass units, with a cycle time of approximately 1.5 s. The eluent was delivered with a system from Varian 9010 coupled with a Varian 9065 Polychrom and an UV detector set at 268 nm. Ten μl were injected into the HPLC column.

3. Results and discussion

Previous studies concerning the quantification of conjugated metabolites of propofol were carried out with hydrolysis leading to the liberation of propofol from PG and 1–4 quinol from 4-QS, 1-QG and 4-QG [2,8,14]. This methodology presents two disadvantages for 1–4 quinol. First, it is impossible to determine the relative proportion of 4-QS, 1-QG and

4-QG. 1-QG and 4-QG could also be metabolized by different UDP-Glucuronyl transferases, as previously described for PG [1,13]. Second, 1–4-quinol may be transformed by tautomeric equilibrium into 1–4-quinone which could be missed by the analytical detection (see below).

3.1. In vitro study

Since phase II metabolites of propofol were not available as pure compounds, glucuronide metabolites were generated by rabbit, rat and human liver microsomes. 4-QS metabolite was obtained from rat cytosols since this metabolite is produced mainly in this species [10]. For each species, assays were carried out on propofol or 1–4-quinol. It was hypothesized that scaling up the time of incubation mixture would proportionally increase the production of glucuronide or sulphate metabolites.

Original chromatographic condition, with UV detection was a modification from Ogata's work concerning conjugated of phenolic compounds [30]. Depending on the species, this method detected only one or two peaks (which increased with the incubation time) when propofol or 1–4-quinol, respectively, were used as substrate. An enzymatic or acidic hydrolysis was performed on the incubation mixture, leading to a partial or a total disappearance of the previous synthesized compounds. These different incubation mixtures were used as samples for optimization of chromatographic conditions.

3.2. HPLC optimization

Ogata and co-workers used a quaternary mobile phase for glucuronic acid and sulphate conjugation of phenolic compounds [30]. The composition of this mobile phase was optimized and methanol was replaced by acetonitrile. A linear gradient was used, to improve the separation, the peak shape and the retention of propofol as well as the phase I and II metabolites. Moreover, a Spherisorb 5 μm ODS 2 stationary phase was preferred. Mobile phases containing organic, inorganic buffer or ion-pairing agents were avoided, since the LC–MS apparatus should be employed for ultimate identification of different metabolites. Unlike previous assays concerning the detection of propofol, PG or 1–4-quinol, isocratic elution was not used [3,13]. This elution mode did not permit the separation of glucuronide, and retention times (t_R) of propofol, 1–4-quinol and 1–4-quinone were dramatically increased with broad peaks. All studies on chromatographic separation of propofol conjugates were performed with the gradient elution mode permitting the separation of different compounds [1,5,7,9,10,28].

Finally, chromatographic conditions described in this study allowed the separation of four peaks scaling with time and obtained from incubation mixtures. In this way, one peak was detected with a t_R equal to 16.3 min when propofol was used as a substrate in microsomes incubation. One peak was detected with a t_R equal to 4.7 min when 1–4-quinol was used as a substrate in cytosolic incubation. Finally, two peaks were detected with t_R equal to 9.3 and 10.5 min, respectively, when 1–4-quinol was used as a substrate in microsomal incubation. Unlike the results of Vree et al., the mobile phase described in this study allows the separation of propofol and 1–4-quinone [28].

3.3. Urine specimen

With optimized chromatographic conditions, several human urine samples from patients undergoing anaesthesia with propofol were analyzed. Neither propofol nor 1–4-quinol (or 1–4-quinone) were detected in these specimens. After enzymatic or acidic hydrolysis, peaks with t_R at 4.7, 9.3, 10.5 and 16.3 min decreased or disappeared, and peaks corresponding to propofol, 1–4-quinol and 1–4-quinone were detected (identification by comparing t_R in the UV spectrum with standard spectra).

Urine samples before and after hydrolysis were then analyzed on a a LC–APCI–MS device. Solution containing propofol, 1–4-quinol, thymol, phenol and phenyl-β-D-glucuronide were used for optimization of APCI–MS parameters. The mass spectra of the compounds examined at 4.7, 9.3, 10.5 and 16.3 min in full scan mode are shown in Fig. 2, when the cone voltage was set to −30 and −60 V. The LC–APCI–MS of the 4.7-min peak, at −30 V cone voltage (Fig. 2A), revealed one fragment, m/z 273.2 (M–H). At −60 V cone voltage two fragments (Fig. 2B), m/z 273.2 and m/z 193.2 (M_{quinol}–H), were observed. Peaks at 9.3 and 10.5 min and −30 V cone voltage

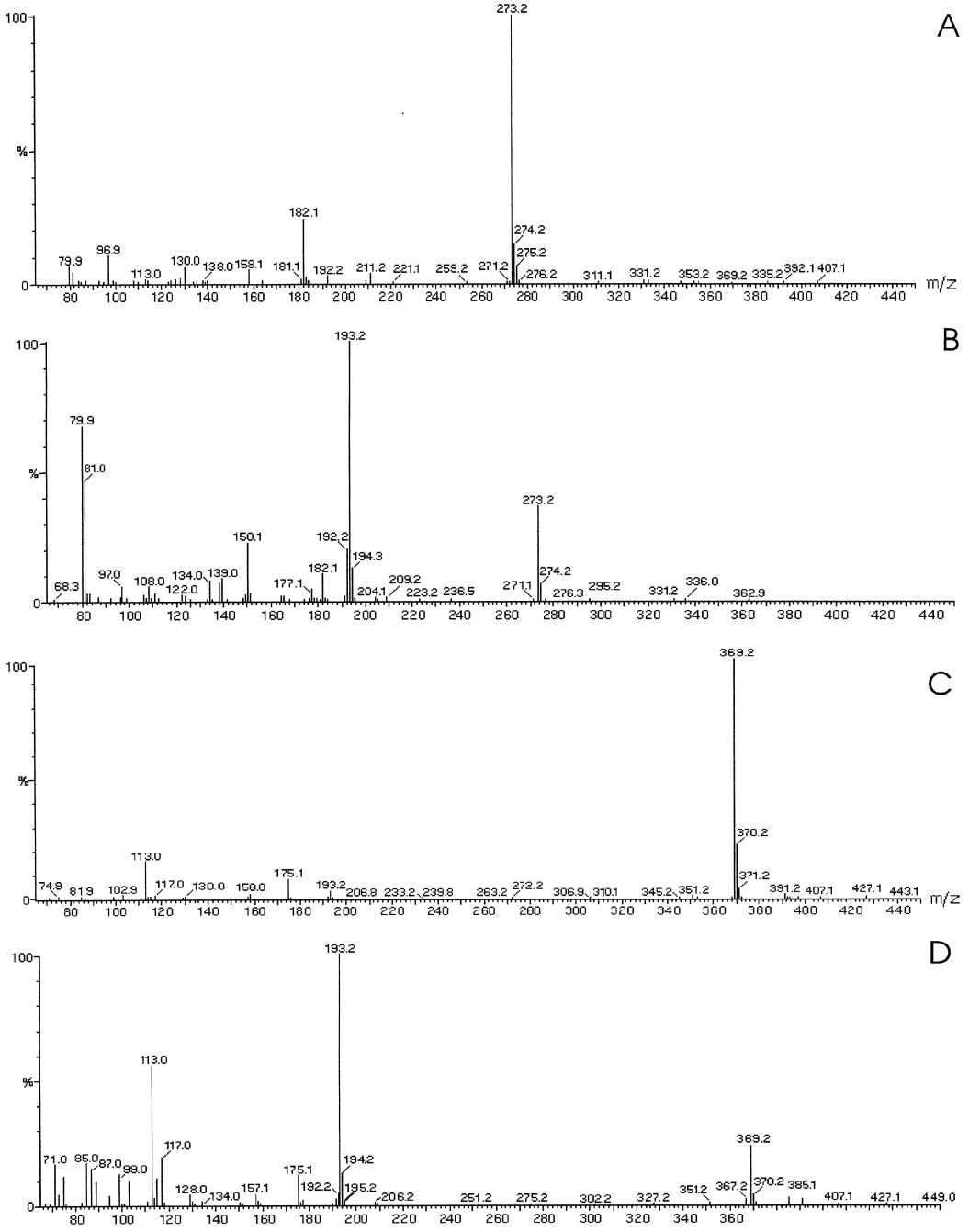

Fig. 2. Mass spectra of 4-quinol sulphate (retention time 4.7 min) with cone voltage at −30 V (A) and −60 V (B). Mass spectra of 1-quinol glucuronide (retention time 9.3 min) with cone voltage at −30 V (C) and −60 V (D). Mass spectra of 4-quinol glucuronide (retention time 10.5 min) were identical. Mass spectra of propofol glucuronide (retention time 16.3 min) with cone voltage at −30 V (E) and −60 V (F). Analytical conditions are described in the text.

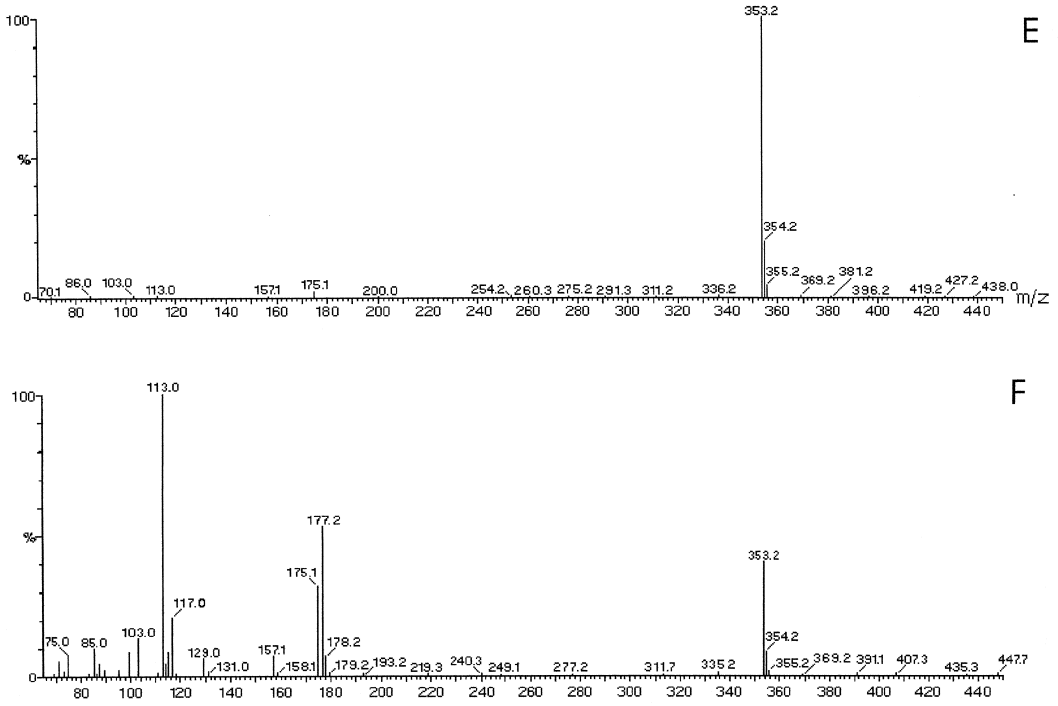

Fig. 2. (*continued*)

(Fig. 2C), revealed one fragment, m/z 369.2 (M–H), and at −60 V cone voltage these two peaks revealed two fragments m/z 369.2 and m/z 193.2 (M_{quinol}–H) (Fig. 2D). At −30 V cone voltage (Fig. 2E), the peak at 16.3 min showed one fragment, m/z 353.2 (M–H), and two fragments m/z 353.2 and m/z 177.2 ($M_{propofol}$–H) at −60 V cone voltage (Fig. 2F). It was concluded that compounds at 4.7 and 16.3 min were 4-QS and PG, respectively, and the two other peaks at 9.3 and 10.5 min corresponded to quinol glucuronides on position 1 or 4. Identification of these two metabolites was impossible with analytical tools used in this study since APCI–MS detection provided the same spectrum for both metabolites. Using LC–MS–MS, the Vree et al. method was not able to identify 1-QG and 4-QG [28].

Identification of these two peaks was based on results obtained from in vitro data and comparison with previous metabolite studies in the literature. During hydrolysis studies it was observed that compound corresponding to a t_R equal to 9.3 min did not undergo hydrolysis with β-glucuronidase, while the peak at 10.5 min totally disappeared in the same conditions. Simons et al. previously made the same observation. They concluded that the glucuronide group of 1-QG was sterically hindered by isopropyl groups in positions 2 and 6, and that 4-QG was not hindered [10]. Microsomal incubations from rabbit samples have shown two peaks at 9.3 and 10.5 min (main) and only one peak at 10.5 min has been detected in rat liver samples. These results were consistent with those previously obtained in vivo by Simons et al., who identified 1-QG only in rabbit and 4-QG in both species [10]. From these data, it was concluded that 1-QG and 4-QG correspond to the peak at 9.3 and 10.5 min, respectively (Fig. 3).

3.4. Detection mode

UV detection was mainly used in this study, in scan mode or with selective wavelengths at 230 and 270 nm, (as usually set for propofol) [4,8,11,12].

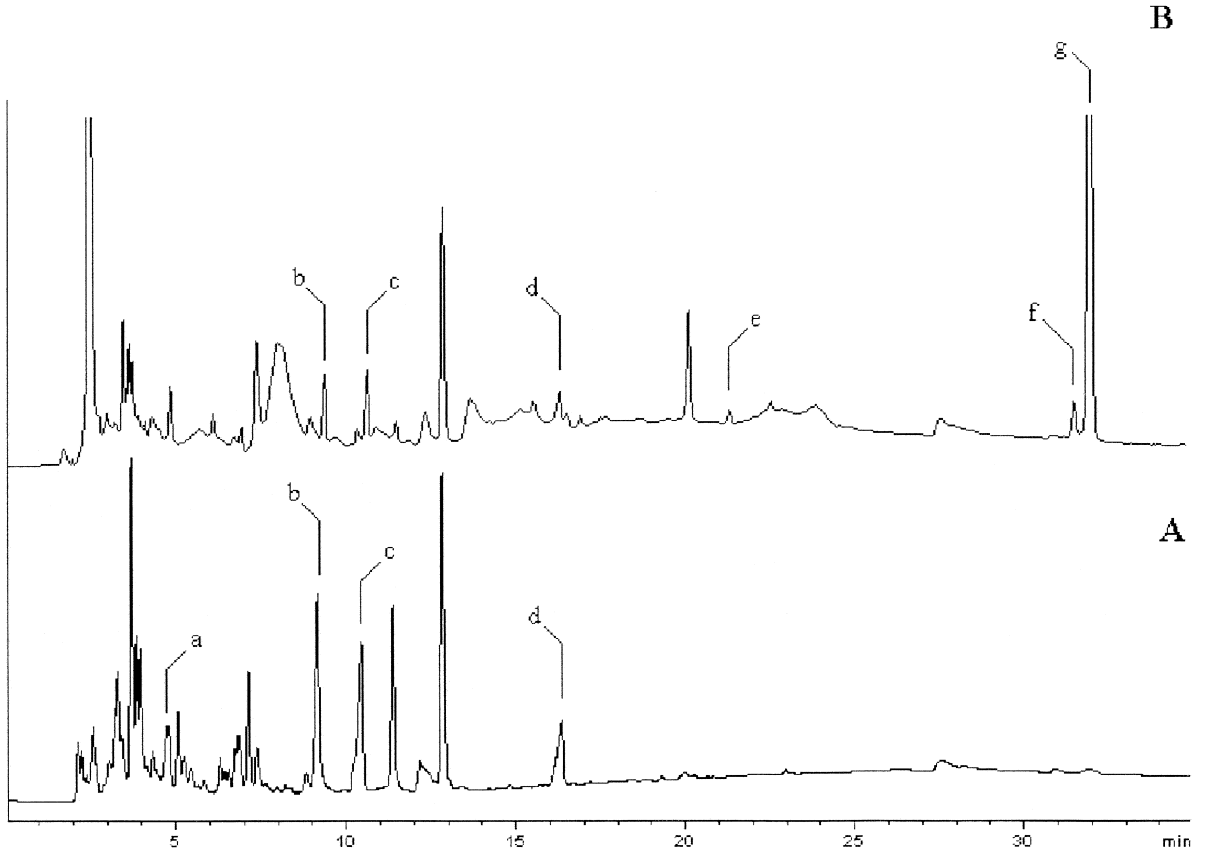

Fig. 3. Chromatogram of urine sample after extraction procedure with UV detection at 270 nm before (A) and after partial chemical hydrolysis (B). (a) 4-QS; (b) 1-QG; (c) 4-QG; (d) PG; (e) 1–4-quinol; (f) propofol; (g) 1–4-quinone.

With UV detection, all metabolites (phase I and II) described in human were identified (Fig. 3). However, in a few urine samples, detection of 4-QS was impossible since an interfering compound was present. Although the extraction procedure improves the selectivity of the assay, this problem was not resolved. In fact, the signal was monitored using the wavelength ratio $\lambda_{220\ nm}/\lambda_{245\ nm}$. In this case, 4-QS was resolved in all urine samples tested, except for the detection of 1–4-quinone. Note that the method was sensitive and selective enough to detect all the compounds (except for 4-QS in urines where an interfering compound was present) with UV at 230 nm and without an extraction procedure.

Fluorescence detection was also performed with the same chromatographic conditions as for UV. For each compound, maximum excitation and emission wavelengths were determined (Table 1). Chromatograms of human urine before and after chemical hydrolysis are shown in Fig. 4, using excitation and

Table 1
Maximum excitation and emission wavelengths of propofol and its metabolites

Compound	λ_{max}	
	Excitation (nm)	Emission (nm)
4-QS	274	318
1-QG	274	318
4-QG	274	322
PG	266	306
Quinol	265	329
Propofol	265	306
Quinone	–	–

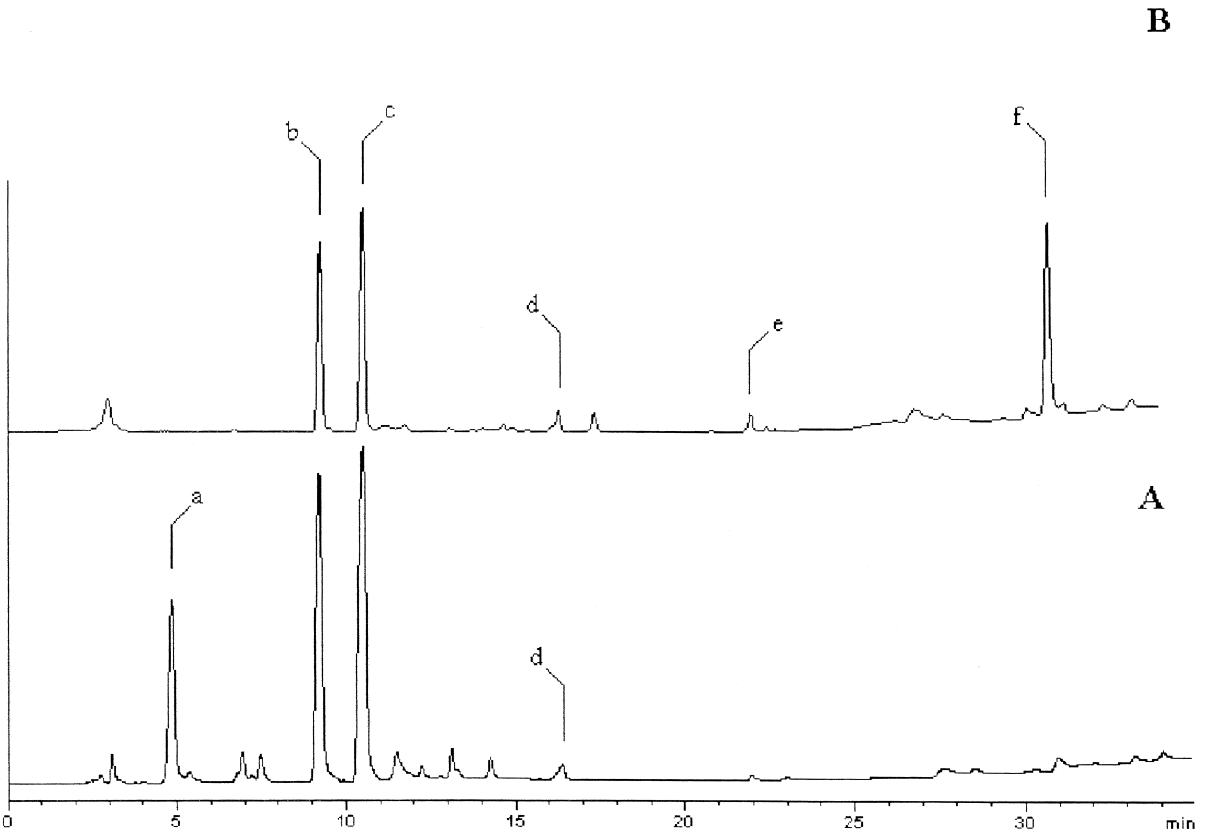

Fig. 4. Chromatogram of urine sample after extraction procedure with fluorescence detection before (A) and after partial chemical hydrolysis (B). (a) 4-QS; (b) 1-QG; (c) 4-QG; (d) PG; (e) 1–4-quinol; (f) propofol. This chromatogram was performed with the same aliquots than those used for Fig. 3.

emission wavelengths at 276 and 310 nm, respectively. Concerning 4-QS, 1-QG and 4-QG, propofol and 1–4-quinol, fluorescence detection provided a better response than UV at 270 nm. Unlike other assays, PG could be detected with fluorescence [4,28]. However, the response was poor and similar to the response obtained with UV at 270 nm. Unlike 1–4-quinol, 1–4-quinone did not give fluorescence response (Fig. 4).

To complete the study, electrochemical detection was also evaluated. For this detection mode, the mobile phase was modified to introduce an electroactive component. The pump system on our chromatographic device was not able to perform gradient elution, so two different isocratic mobile phases were used: one for propofol and phase I metabolites, and the other one for phase II metabolites. In oxidation mode, 4-QS, 1-QG, 4-QG, 1–4-quinol and propofol were detected using coulometric detection. However, PG and 1–4-quinone were not detected. To compare the response of compound with close structure, phenol and phenyl-β-D-glucuronide were injected into HPLC. As described for propofol, phenol was detected, whereas phenyl-β-D-glucuronide was not detected. Using the mobile phase described previously in this study, t_R values of 1-QG, 4-QG, 4-QS were 4.0, 4.9 and 7.2 min, respectively (Fig. 5).

Consequently the indirect quantification of 4-QS, 1-QG and 4-QG by 1–4-quinol after total hydrolysis seems uncertain with fluorescence and electrochemical detection modes, since the production of 1–4-quinone could lead to an erroneous result. Moreover, electrochemical detection does not allow the identification of PG.

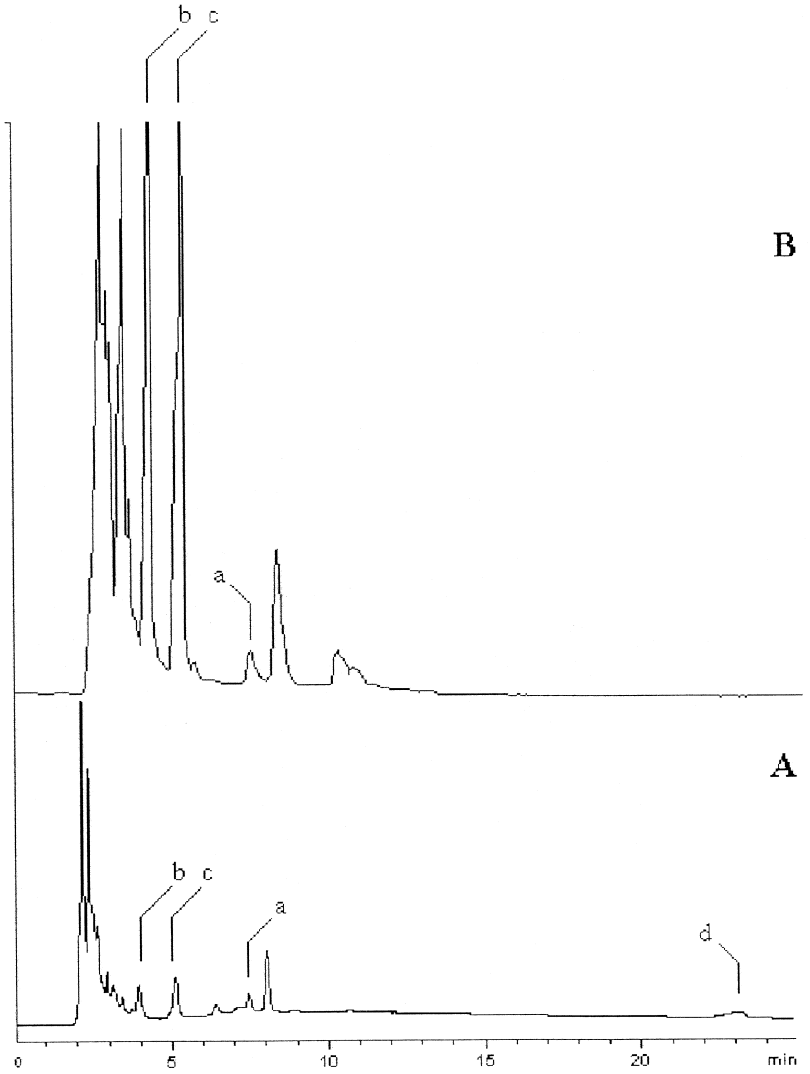

Fig. 5. Chromatogram from urine extract with UV detection (A) and electrochemical detection (B). The mobile phase consisted of acetonitrile–25 m*M* phosphate buffer, pH 3.8 (25:75, v/v). For electrochemical detection, electrode potential for detector 2 was set at +650 mV (100 μA full scale) and UV was set at 270 nm.

4. Conclusion

Simple chromatographic conditions described in this study allowed us to separate in human urine, in a single run, phase I and phase II (glucuronide and sulphate conjugates) of propofol. It is the first time, to our knowledge, that a simple analytical technique is described, where all the propofol metabolites known in human are separated simultaneously. The main results described in this paper partially confirm those recently published by Vree et al. who present an assay to quantify the propofol glucuroconjugate [28]. The methodologies are different between these two studies, particularly the use of in vitro assays to generate the conjugate metabolites in the present study. Only qualitative analyses have been accomplished in our study because we cannot propose a reliable quantification, since a high quality purification of the different conjugate metabolites was not obtained. However, the separation and the identifica-

tion of 4-QS was achieved. Finally, after the comparison of several HPLC detectors, we conclude that detection of conjugate metabolites can be performed with UV, fluorescence or electrochemical detection, except for PG in this last detection mode. Only UV detection allowed the identification of all phase I and phase II propofol metabolites.

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